

THE BIOLOGICAL BULLETIN

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER THIGMOTRICHA)

I. HYPOCOMINA TEGULARUM SP. NOV. AND CREBRICOMA GEN. NOV.

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INTRODUCTION

The genus *Hypocomina* was proposed by Chatton and Lwoff (1924) to include a single species, *Hypocoma patellarum* Lichtenstein (1921), parasitic on the pallial branchiae of *Patella caerulea* L. Subsequently, Raabe (1934) described as *Hypocomina carinata* a ciliate from *Mytilus edulis* L. which Jarocki (1935) pointed out obviously does not belong to the genus *Hypocomina*. Raabe used the name *Hypocomina carinata* again in 1938, however, and no literature has come to my attention in which the form in question is assigned to another genus. In the present paper I will describe as *Hypocomina tegularum* sp. nov. an ancistrocomid ciliate from the ctenidium of *Tegula brunnea* (Philippi); and on the basis of my studies on the morphology of a ciliate which I presume to be identical with "*Hypocomina*" *carinata*, I will establish the position of Raabe's species in a new genus, *Crebricoma*.

HYPOCOMINA TEGULARUM SP. NOV.

(Plate I, Figs. 4, 5)

The body is elongated and compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from $26\ \mu$ to $36\ \mu$, in width from $12\ \mu$ to $17\ \mu$, and in thickness from $9\ \mu$ to $12\ \mu$, averaging about $31\ \mu$ by $15\ \mu$ by $11\ \mu$. As seen in dorsal view the curvature of the left margin of the ciliate is a little more pronounced than that of the right margin, although this asymmetry is not conspicuous and in most fixed specimens is barely apparent. The body is widest near the middle and is rounded posteriorly. The anterior end is attenuated, bent ventrally, and truncate at the tip. The reduced ciliary system, to be described presently, is disposed in a shallow, relatively flat depression occupying the anterior half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic field are convex.

A contractile suctorial tentacle enables the ciliate to attach itself to the epithelial cells of the ctenidium of the host and to suck out their contents. I have not yet succeeded in determining the exact nature of the tentacle in the ancistrocomid ciliates which I have examined and the accounts of other authors regarding its structure are likewise unsatisfactory. In *Hypocomina tegularum*, it appears to be a membranous tube-like extension of the attenuated anterior end which is protracted and contracted at will. When fully extended the tentacle is about 3μ in length and $1-1.5\mu$ in diameter. It is hoped that further studies on ancistrocomid ciliates will shed some light on the problem of the structure of this interesting organelle.

The internal tubular canal continuous with the suctorial tentacle is difficult to study in the living ciliates, but in material stained with iron hematoxylin or alum hematoxylin it can usually be traced posteriorly for a distance equal to about one-third the length of the body (Plate I, fig. 4, c). In most individuals it is directed obliquely to the right. In fixed specimens the extreme anterior portion of the canal is approximately $1-1.5\mu$ in diameter, while the remainder of it appears to be swollen and to have the form of a poorly defined clear area. Lichtenstein (1921) interpreted the comparable swelling of the canal in *Hypocomina patellarum* to be a "balle alimentaire." Jarocki (1935) attributed the clear area described by Raabe (1934) behind the tentacle of *Hypocomella macomae* and "*Hypocomina*" *carinata* to hyperdistension of the canal due to unequal infiltration by the fixing reagent or as a result of its action upon moribund, partly plasmolyzed individuals. It is interesting to note in this connection, however, that some ancistrocomid ciliates rarely show this phenomenon upon fixation and the canal can be traced to within a short distance of the posterior end of the body.

The delicate thigmotactic cilia of *Hypocomina tegularum* are about $6-7\mu$ in length and are disposed in nine longitudinal rows. All the rows originate about 3μ or 4μ posterior to the base of the suctorial tentacle and are about one-half the length of the body (Plate I, fig. 4). The first five rows from the right side of the ciliate are appreciably longer and sometimes appear to lie closer together than the other four rows. Chatton and Lwoff (1924) reported that in *Hypocomina patellarum* a ridge-like eminence (carène) divides the ciliary area into two unequal fields. These authors did not, however, state how many of the ten ciliary rows described by Lichtenstein (1921) for *H. patellarum* are in each complex and did not give any facts concerning the relative lengths of the rows comprising the two fields. In *Hypocomina tegularum* I have not discerned an unmistakable eminence separating the five rows on the right from the four rows on the left, although in some living and fixed individuals the distance between the distal portions of the fifth and sixth rows is somewhat greater than the distance between the other rows.

EXPLANATION OF PLATE I

FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. $\times 1130$.

FIGURE 2. *Crebricoma carinata* (Raabe) comb. nov. Dorsal aspect, from life.

FIGURE 3. *Crebricoma carinata* (Raabe) comb. nov. Lateral aspect from right side, from life.

FIGURE 4. *Hypocomina tegularum* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. Drawn with aid of camera lucida. $\times 1870$.

FIGURE 5. *Hypocomina tegularum* sp. nov. Lateral aspect from left side, from life.

c = internal tubular canal, cv = contractile vacuole, fv = food vacuole, ma = macronucleus, mi = micronucleus.

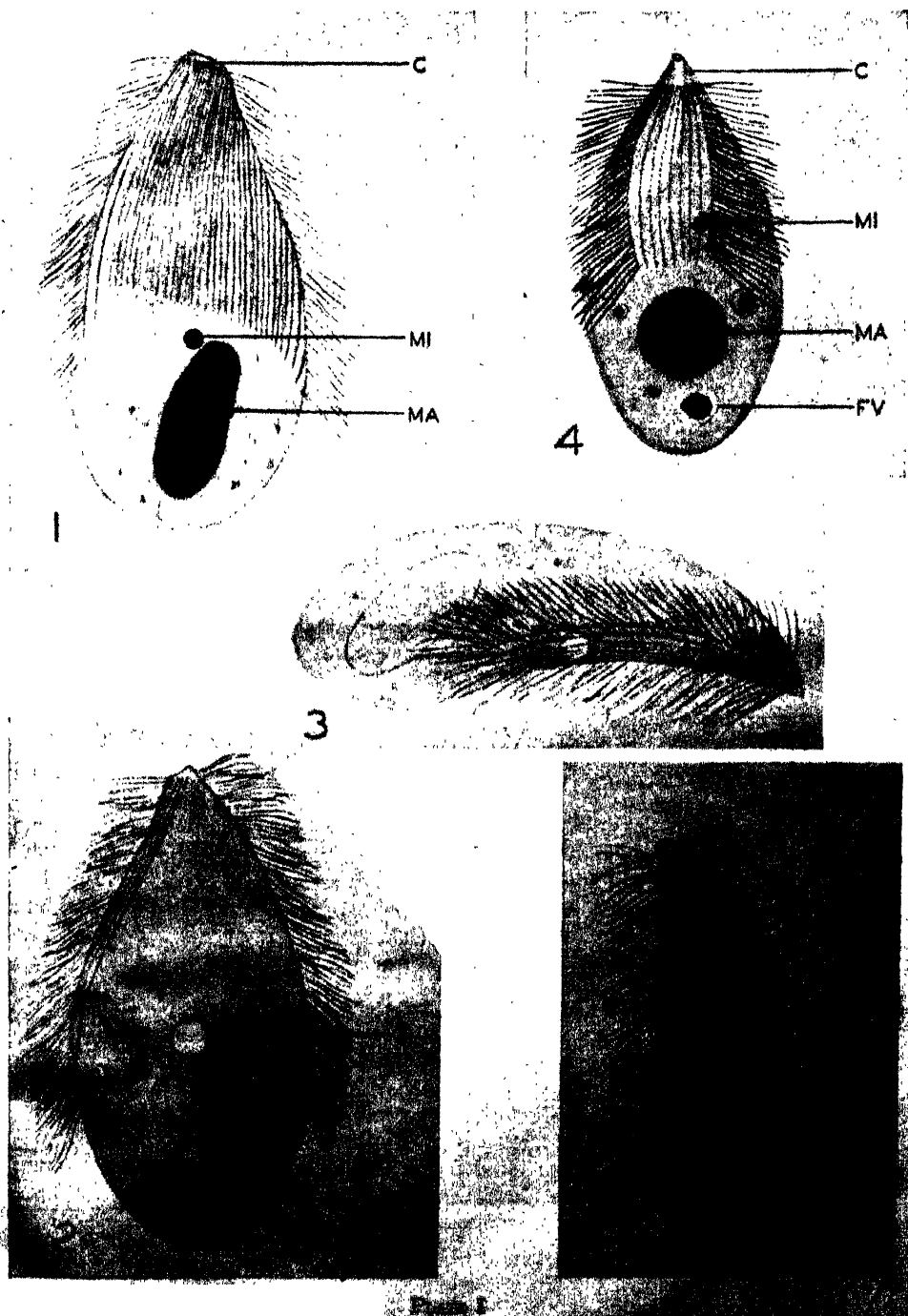


FIG. 1

The cytoplasm is colorless and contains numerous food inclusions and refractile granules. The refractile granules are apparently lipid droplets, as they are dissolved out by toluol used for clearing following staining. One or more large food vacuoles (Plate I, fig. 4, fv) are usually present in the posterior part of the body. The contractile vacuole (Plate I, fig. 5, cv) lies near the middle of the body and opens to the exterior on the ciliated ventral surface. I have not detected a permanent opening in the pellicle.

The spherical macronucleus (Plate I, fig. 4, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or alum hematoxylin the chromatin appears to be aggregated into many large, round granules. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the macronucleus ranged from $5.5\ \mu$ to $7\ \mu$.

The spherical or ovoid micronucleus (Plate I, fig. 4, mi) is usually situated anterior to the middle of the body. It is very difficult to detect in living individuals. In stained preparations the chromatin is dispersed into granules of varying size and shape lying for the most part near the periphery. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the diameter of the micronucleus ranged from $1.6\ \mu$ to $2.2\ \mu$.

I found *Hypocomina tegularum* to be present in small numbers on the ctenidium of two of three specimens of *Tegula brunnea* (Philippi) which I collected at Carmel Bay, California, in September, 1944.¹ Since that time I have had the opportunity to examine a large number of individuals of *Tegula brunnea* from localities near Moss Beach and Dillon Beach, California, but found none to be infested by *Hypocomina tegularum*.

Hypocomina tegularum sp. nov.

Diagnosis: Length $26\ \mu$ – $36\ \mu$, average about $31\ \mu$; width $12\ \mu$ – $17\ \mu$, average about $15\ \mu$; thickness $9\ \mu$ – $12\ \mu$, average about $11\ \mu$. The anterior end is attenuated, bent ventrally, and provided with a contractile suckorial tentacle continuous with an internal tubular canal. The ciliary rows are nine in number and are disposed in a shallow depression occupying the anterior half of the ventral surface. The first five rows from the right are slightly longer than the other four rows. The macronucleus is spherical and is situated in the posterior half of the body. The micronucleus lies anterior to the middle of the body. Ectoparasitic on the ctenidium of *Tegula brunnea* (Philippi) (Carmel Bay, California). Syntypes are in the collection of the author.

CREBRICOMA GEN. NOV.

CREBRICOMA CARINATA (RAABE) COMB. NOV.

(Figure 1. Plate 1, Figs. 1-3)

I have distinguished two species of ancistrocomid ciliates parasitic on the epithelium of the palps and ctenidial filaments of *Mytilus edulis* L. from various localities in San Francisco Bay. One of these is *Hypocomoides mytili* Chatton and Lwoff; the other I presume to be identical with "*Hypocomina*" *carinata* Raabe, for which I propose a new genus, *Crebricoma*. My observations on the morphology

¹ I am indebted to Dr. D. T. MacDougal for his kindness in arranging my trip to Carmel which made possible the collection of the original material of the ciliate described herein.

of this species permit me to augment the original description given by Raabe. It seems advisable, therefore, to present herein a revised description of *Crebricoma carinata*.

The body is elongated and somewhat compressed dorso-ventrally. Fifteen living individuals taken at random ranged in length from 58μ to 71μ , in width from 27μ to 39μ , and in thickness from 22μ to 31μ , averaging about 64μ by 31μ by 25μ . The anterior end is narrowed, bent ventrally, and in dorsal view is seen to end in an oblique truncation, the right side of which projects a little more than the left side (Plate I, figs. 1, 2). The body is widest near the middle and is rounded posteriorly. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the thigmotactic area are convex.

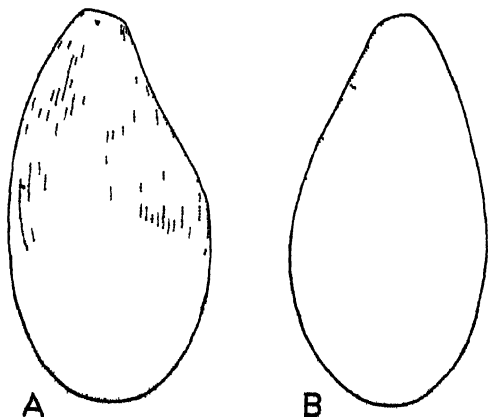


FIGURE 1. *Crebricoma carinata* (Raabe) comb. nov. Distribution of ciliary rows. Semi-diagrammatic representations based on camera lucida drawings of specimens fixed in Schaudian's fluid and impregnated with activated silver albumose (protargol).

A. Ventral aspect, B. Dorsal aspect, C. View of anterior end.

The suckorial tentacle is situated on the right side of the anterior truncation. I am at present unable to make any conclusive statement with regard to the structure and contractile properties of the tentacle in this species. The internal tubular canal continuous with the tentacle cannot be satisfactorily distinguished in living material, but can be demonstrated in some fixed specimens which have been stained with iron haematoxylin. It appears to pass at first dorsally and then ventrally and obliquely to the right. I have not succeeded in tracing it posteriorly for more than a short distance. A broad lighter area behind the anterior part of the canal is frequently observed in fixed individuals.

As described by Raabe, the ciliary system of *Crebricoma carinata* consists of about twenty closely-set rows bordered on the right by two longer and more widely spaced rows and on the left by three such rows. According to my observations, however, the more widely-spaced rows on the left side do not separate themselves from the closely-set rows. The number of rows in the anterior

of *C. carinata* is very difficult to determine, since some of the rows on the left are lateral in position and several of them originate on the dorsal surface. In one individual impregnated with activated silver albumose I counted thirty-four rows and in another individual, thirty-six rows. In all specimens which I have examined carefully the number of ciliary rows exceeded thirty-two.

The two widely-spaced rows on the right side of the thigmotactic field originate on the ventral surface near the base of the suckorial tentacle. The outer row is the longer and is about two-thirds the length of the body (Fig. 1A). All the rows of the second complex, with the exception of three or four rows on the extreme left which are about as widely-spaced as the two long rows on the right side, are very closely-set and are one-half to two-thirds the length of the body, becoming progressively longer toward the left. Several of the rows on the left side of the thigmotactic field originate on the left lateral margin or on the dorsal surface (Fig. 1B; Plate I, fig. 2). The arrangement of the ciliary rows at the anterior end thus forms an incomplete suture (Fig. 1C) reminiscent of the anterior field of other holotrichous ciliates.

The cilia of *Crebricoma carinata* are about 10–11 μ in length. When the organism is attached to the epithelium of the palps or ctenidial filaments of the host the cilia usually exhibit only a sluggish movement. Occasionally one or two small groups of cilia near the anterior end beat energetically. When dissociated from the host *C. carinata* swims actively and its cilia beat metachronously.

Raabe proposed the specific name *carinata* in allusion to a keel-like prominence on the dorsal surface. I have seen such prominences on seriously shrunk fixed specimens of *Crebricoma carinata* and on some living individuals which were obviously undergoing plasmolysis. I have not, however, been able to distinguish them on ciliates freshly removed from the host. Raabe also described a sunken space between the keel-like prominence and the outermost ciliary row on the right side, which he said "vermutlich dem von Chatton und Lwoff bei anderen Hypocomiden beschriebenen 'vestige de frange adorale' entspricht." I do not believe such a depression exists in normal individuals, although I have observed groove-like and slit-like depressions form on the lateral and dorsal surfaces of moribund specimens of *Crebricoma carinata*, as well as *Hypocomidex mytili*.

The cytoplasm is colorless and contains a few small refractile granules, which presumably are lipid droplets, and food inclusions. I have not discerned any large food vacuoles in this species. The contractile vacuole (Plate I, fig. 2, cv) opens to the exterior on the ventral surface near the middle of the body. There appears to be no permanent opening in the pellicle.

The sausage-shaped or ovoid macronucleus (Plate I, fig. 1, ma) is situated in the posterior half of the body. In preparations stained with iron hematoxylin or the Feulgen nuclear reaction the chromatin is aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. Viewed dorsally, the longitudinal axis of the macronucleus is ordinarily placed obliquely to the longitudinal axis of the body. In most specimens the anterior end of the macronucleus is directed dorsally while the posterior end is directed ventrally. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from 13.4 μ to 24.3 μ and in width from 5.6 μ to 11.7 μ .

The spherical micronucleus (Plate I, fig. 1, mi) is readily detected in the living state. It is commonly situated near the dorsal surface close to the anterior end of

Schaeffer (1926, 1938a) maintains that Wilson's amoeba represents Roesel's (1755) "der kleine Proteus," and should therefore be called *Chaos chaos* Linnaeus. But Roesel's description is entirely inadequate to establish a species or even a genus. He undoubtedly had an amoeboid form, but it is impossible to find out the exact structure of his "Proteus." He mentions granules (*Körnern*) but says nothing of vacuoles, which in the giant amoeba are much larger than the crystals (granules). Schaeffer seems to base his conclusion mainly on the size and shape of the "Proteus." He contends that Roesel was a reliable investigator and that his figures may therefore be credited with general accuracy. As Schaeffer (1926) points out, "Roesel states the natural size of his amoeba in the rounded (spherical?) form to have been the same as figure A, which measures about 1660 μ in diameter." Yet Schaeffer (1938a) says, "*Chaos chaos* has a distinctive size range which fluctuates around 500 μ diameter." Thus, Schaeffer himself gives the diameter of the giant amoeba as about $\frac{1}{3}$ that of Roesel's "Proteus." There are also striking differences between Roesel's figures and description of a binary fission in his "Proteus" and Schaeffer's (1938b) photographs and account of the "3-daughter division of the giant amoeba."

Mast and Johnson (1931) review the data presented by Roesel and express the opinion that the latter possibly was dealing with a myxomycete. Rice (1945) states, "It is impossible to ascertain the exact structure of Roesel's 'der kleine Proteus.'" Thus since both the generic and specific names, *Chaos chaos*, are based on Roesel's inadequate description, they are not valid.

Hegner and Taliaferro (1924) in referring to Wilson's large amoeba use the name *Amoeba carolinensis* for it, though they give no reason for doing so. The present investigation adds evidence to support the use of this name. Therefore, *Amoeba carolinensis* (Wilson, 1900) Hegner and Taliaferro, 1924 is considered to be the correct name of the organism used in these experiments.

CYTOLOGY AND PHYSIOLOGY

MATERIALS AND METHODS

The original stock culture of *Amoeba carolinensis* was procured from the General Biological Supply House, Chicago, Illinois, in November 1944. About every two weeks subcultures were made in the following manner: Spring water containing five wheat grains per 100 cc. of water was boiled for a few minutes and left uncovered for several days. One or two of the grains of wheat were put into a butter dish and about 90 cc. of the boiled water was poured in, giving a depth of $\frac{3}{4}$ inches. The amoebae along with food organisms, which consisted chiefly of rotifers, *Chilomonas paramecium*, *Colpidium colpoda*, and *Paramecium caudatum*, were then added. In the best cultures a water mold grew on the wheat grains and many amoebae often were found among the hyphae of the mold. These stock cultures had been maintained for a little more than four months when the experiments began.

In addition to the wheat culture medium (W. C. M.) a hay medium (H. C. M.) was made as follows: 10 grams of chopped timothy hay were added to 1000 cc. of spring water and boiled for about 15 minutes. The solution was filtered to remove the solid particles and then put into test tubes with cotton stoppers and autoclaved on two successive days at 10 pounds pressure. From these test tubes the medium

was taken to start new cultures during the experiments. After trying various concentrations of this hay medium it was found that the amoebae and food organisms remained healthier and increased more rapidly if the stock solution was diluted with four parts of boiled and aerated spring water.

Cultures of *Chilomonas paramecium* and *Paramecium caudatum* were established in both the wheat and the hay culture media to serve as food organisms for the amoebae during the experiments. Subcultures were made from these about every two weeks.

Forty specimens of *Amoeba carolinensis* were washed six times in boiled spring water. Ten were transferred to two stender dishes (five to each dish) containing paramecia in W. C. M. In a similar manner ten were transferred to two stender dishes containing chilomonads in W. C. M.; ten, to two stender dishes containing paramecia in H. C. M.; and ten, to two stender dishes containing chilomonads in H. C. M. Each day the cultures were inspected and the supply of food organisms was replenished if it was low. The amoebae were counted daily for seven-day periods and then on the seventh day (in some cases a few days later) new cultures were made from the ones of the preceding week, so that by the end of the experiment most of the amoebae had been growing in one kind of medium and feeding on one kind of food organism for at least 46 days. With the exception of two cultures, the pH was taken on each culture on or after the seventh day. During the experiment, observations as to the relative size and form of the amoebae were made; some of the amoebae in each culture were examined in hanging drops under the compound microscope; and at the end of the experiment photomicrographs were taken of a few amoebae in the wheat-culture medium.

RESULTS

After the first two weeks there were marked differences in the rates of reproduction, especially between those amoebae grown in W. C. M. and fed on paramecia and the other cultures, as can be seen in Table I. During the first two weeks there seems to be a period of adjustment when the reproduction rates of the different cultures do not vary significantly. However, during the third to sixth weeks the amoebae grown in W. C. M. and fed on paramecia increased rapidly from 10 to 45 during the sixth week. The other cultures showed little or no growth.

The hydrogen-ion concentrations remained rather constant. The pH's varied from 7.0 to 8.1, with that of the H. C. M. containing paramecia usually a little higher than the others. The room temperature varied from about 20° to 26° C.

There was no significant difference in size of the amoebae in the various cultures, but there was a difference in form. Those growing in W. C. M. and fed on paramecia were often somewhat disc-shaped with short, blunt pseudopods radiating from all sides as shown in Figure 1a. Others were usually monopodal or bipodal with blunt pseudopods as shown in Figure 1b. Clumping of the disc-shaped amoeba was observed five or six times.

The amoebae in W. C. M. fed on chilomonads were more flattened, with thinner pseudopods which were often at right angles to the main part of the body and frequently branched (Fig. 1c). The pseudopods often had little knob-like swellings at their distal ends (Fig. 1d). The amoebae in these cultures occasionally were monopodal in form.

TABLE I

Effect of culture media and food organisms on the reproduction rates of *Amoeba carolinensis*. At the beginning of each week amoebae were put into new cultures corresponding to the conditions under which they previously had been growing, so that the end results represent cumulative effects. Since there always were two cultures of each type, the pH's of both cultures are given.

	Wheat medium with paramecia				Wheat medium with chilomonads				Hay medium with paramecia				Hay medium with chilomonads			
	Number of amoebae		% in- crease	pH	Number of amoebae		% in- crease	pH	Number of amoebae		% in- crease	pH	Number of amoebae		% in- crease	pH
	First day	Sev- enth day			First day	Sev- enth day			First day	Sixth day			First day	Sev- enth day		
First week	10	17	70%	7.5 7.6	10	22	120%	7.5 7.5	10	18	80%	7.7 7.8	10	11	10%	7.5 7.5
Second week	10	15	50%	7.6 7.6	9	14	55½%	7.5 7.6	10	11 Sev- enth day	10%	8.0 7.9	10	10	0%	7.8 7.7
Third week	10	30	200%		10	10	0%	7.8 7.9	10	16	60%	8.0 8.1	10	10	0%	8.0 8.1
Fourth week	10	48	380%	7.0 7.1	10	10	0%	7.6 7.6	10	10	0%	7.6 7.4	10	11	10%	7.9 8.0
Fifth week	10	57	470%	7.5 7.4	10	13	30%	7.6 7.6	10	5	-50%	7.6 7.4	10	10	0%	7.6 7.6
Sixth week	10	45	350%	7.3 7.4	10	12	20%	7.4 7.4	5	4	-20%	7.8 7.6	10	10	0%	7.3 7.2

In H. C. M. the animals differed very little from each other as far as external appearance is concerned. They were, for the most part, monopodal or bipodal. Sometimes, however, those with the chilomonad diet exhibited the branched form characteristic of the amoebae in W. C. M. with chilomonads. Those amoebae with the paramecium diet were usually slightly larger than the ones feeding on chilomonads, and during the last two weeks one specimen grew to be the largest amoeba in any of the cultures. Very large vacuoles were apparent in the amoebae in H. C. M. with the paramecium diet, and some individuals were spherical. These last two characteristics are probably associated with degeneration.

In Wilson's description (1900) of this giant amoeba, he mentions minute, elongate, and fusiform crystals in the endoplasm. Wilber (1942) describes the crystals in more detail and says that there are two types: (1) plate-like crystals, and (2) bipyramidal crystals, which are the more numerous. He states that these crystals (presumably both kinds) are formed from food in the food vacuoles. Wilson also mentions spherical bodies 8 μ in diameter and smaller, which resemble oil drops. Wilber gives the size range of these bodies as 2.5-8 μ in diameter, and



FIGURE 1. Photomicrographs of amoebae grown in wheat culture medium. a and b, those with parametia as food organisms. c and d, those with chilomonads as food organisms

concludes (1945) that they are formed from the crystals and the vacuole refractive bodies. He then says that the refractive spherical bodies function as reserve food in the amoeba.

While examining the stock and experimental amoebae under the compound microscope, differences in regard to the bipyramidal crystals and spherical bodies were noted. The plate-like crystals were so few in number that it was difficult to make a valid comparison in regard to their relative numbers and sizes.

In the stock cultures the amoebae had few spherical bodies, the largest of which were about $5\ \mu$ in diameter. The largest bipyramidal crystals were about $2.4\ \mu$ long.

The amoebae grown in W. C. M. with a diet of paramecia showed some larger bipyramidal crystals $2.8\ \mu$ long and a great number of smaller ones. There were few spherical bodies and the largest were only $3.1\ \mu$ in diameter.

Those specimens grown in W. C. M. and fed on chilomonads showed practically the same condition in regard to the bipyramidal crystals. However, there were very many large spherical bodies measuring $8.5\ \mu$ in diameter.

The spherical bodies of the amoebae grown in H. C. M. and fed paramecia were slightly more numerous and a little larger than the ones in the amoebae grown in W. C. M. with a paramecium diet. There were a few larger bipyramidal crystals $4.2\ \mu$ long, more $2.8\ \mu$ long and some smaller ones.

The amoebae grown in H. C. M. with chilomonads showed many large bipyramidal crystals $4.2\ \mu$ long, and the spherical bodies were very large and numerous. Many of the latter were $9.8\ \mu$ in diameter, and some were slightly flattened on one side so that they looked like a three-quarter-full moon.

DISCUSSION

With the exception of size and number of nuclei, *Amoeba proteus* and *A. carolinensis* are very much alike. Schaeffer (1926) states, "My study of this matter (a comparison of *A. proteus* and *A. carolinensis*) leads me to include *diffluens* (*proteus*) in the same genus with *Chaos* (the giant amoeba). These two species, as a matter of fact, resemble each other more closely than most other species within one genus."

From Wilber's (1942) description of the cytology of *A. carolinensis* it is seen that the giant amoeba resembles *A. proteus* very closely in regard to the shape and structure of the nuclei and the cytoplasmic inclusions. Wilber (1945) has also shown that these two forms are very much alike in respect to the function of the nuclei, the formation of the contractile vacuoles, and the formation and function of the spherical bodies.

When the data of these experiments are compared with similar work on *A. proteus*, it is shown that these two amoebae resemble each other in nutritional requirements. The numbers and relative sizes of the crystals and spherical bodies in the amoebae in my experiments agree in general with the results of similar studies by Mast (1939) and Mast and Hahnert (1935) on *A. proteus*. Concerning the numerous spherical bodies in the chilomonad-fed amoebae, Mast and Hahnert say, "This is of considerable importance for, as stated above, it indicates that the neutral red staining droplets found in abundance in *Chilomonas* but not in *Colpidium* function in the formation of the spherical bodies in *Amoeba*." My experiments indicate that this is also the case in *A. carolinensis*.

In examining the data of these nutrition studies it is seen that in general the amoebae with the most numerous and largest crystals and spherical bodies had the lowest reproduction rates. In view of the fact that the spherical bodies serve as reserve food (Wilber, 1945), these experiments indicate that even though chilomonads may be ingested and digested by the amoebae, this diet is not adequate for normal reproduction. The reproduction rates in the different cultures agree with the results of Reynolds (1938) and Williamson (1944) for *A. carolinensis*, but do not correspond with Williamson's data on *A. proteus*.

The relative sizes of the amoebae in the various cultures do not agree very closely with the observations during similar experiments by Mast (1939) on *A. proteus* and Williamson (1944) on *A. proteus* and *A. carolinensis*. In my experiments there was no increase in the size of the amoebae which had eaten ciliates, as found by both these authors, nor did the chilomonad-fed amoebae decrease in size, as found by Williamson.

In regard to the form of *A. carolinensis* while utilizing paramecia as food organisms, the results are in accord with those of Mast and Root (1916) for *A. proteus* and Williamson (1944) for *A. proteus* and *A. carolinensis*.

NUCLEAR DIVISION

MATERIALS AND METHODS

Specimens were taken from the stock cultures and fixed with Carnoy's, Belling's, and Bouin's fixatives. Those fixed with Carnoy's and Belling's fluids were stained with Delafield's and Heidenhain's haematoxylin. Those fixed in Bouin's fixative were stained with Heidenhain's haematoxylin. Some specimens were sectioned at 10μ . Since those fixed with Carnoy's fixative and stained with Heidenhain's haematoxylin showed the nuclear structure best and stained the cytoplasmic inclusions very little, all the remaining work on nuclear divisions was done with these reagents.

In order to secure nuclear divisions, slides were made at various hours of the day. All the specimens showing nuclear division stages were fixed between 5:45 p.m. and 11:00 p.m. and were more or less spherical in form. The experiments are not extensive enough to warrant any statement as to a periodicity of mitosis. This seeming periodicity was probably a result of better selection toward the end of the experiment.

OBSERVATIONS AND DESCRIPTIONS

Resting nucleus

The resting nucleus of *A. carolinensis* (Fig. 2a) is disc-shaped, and measures about $24 \times 10\mu$. Immediately beneath a distinct nuclear membrane and adhering closely to it are darkly staining granules (peripheral granules), the largest of which are about 2μ in diameter. The interior of the nucleus has a finely granular appearance and stains more lightly than the peripheral granules.

Schaeffer (1938) states that all the nuclei of an individual divide at the same time. Yet, nuclei at slightly different stages of division can be found in the same animal.

Prophase

In early prophase the nucleus enlarges apparently by a pulling away of the membrane from the central granules leaving a large endosome about 19μ in greatest diameter. The nucleus now measures $27 \times 15.4\mu$. Most of the peripheral granules are still rather small and stain more darkly than the endosome. Some of them however are slightly larger and less deeply stained than those in the resting nucleus. Faint strands running from the endosome to the periphery are now apparent.

In a little later stage (Fig. 2b and c) the nucleus has become more spherical measuring about $27 \times 22\mu$. Some of the peripheral granules have become loosened

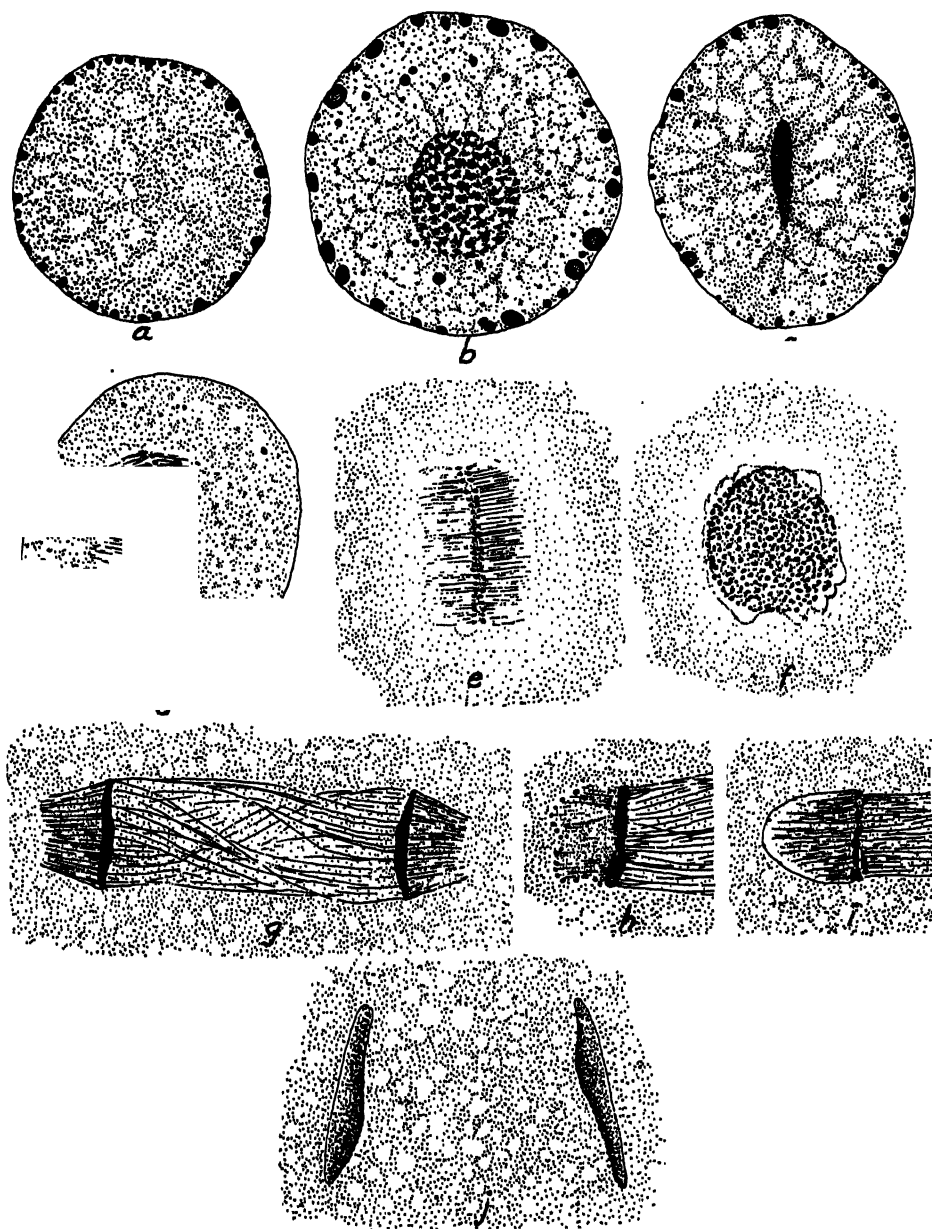


FIGURE 2. Camera lucida drawings of the stages of mitosis in *Amoeba carolinensis*. a. resting nucleus; b. prophase, face view; c. prophase, edge view; d. very late prophase showing chromosomes and spindle forming; e. metaphase, edge view; f. metaphase, polar view; g. late anaphase; h. early telophase, showing one plate with granules at the pole; i. a little later stage, showing one pole with a delicate membrane and granules; j. late telophase with membrane fully formed. All drawings $\times 1660$.

from the membrane, are more spherical, and show a lighter area in the center. The endosome has become smaller and more compact, and now stains about as deeply as the peripheral granules. At this stage the endosome is a thin disc measuring $9.3\ \mu$ in diameter and $1.5\ \mu$ in thickness. The reticulum connecting the endosome with the periphery is more evident now.

Very late prophase (Fig. 2d) shows the chromosomes becoming arranged on the plate which is $14.2\ \mu$ in diameter. Spindle fibers are distinct and the peripheral granules have practically disappeared. A lighter area in the cytoplasm immediately surrounding the nucleus is visible. The nucleus shown in Figure 2d was the only nucleus in the animal in this condition. The remaining nuclei were in metaphase or early anaphase.

Metaphase

At metaphase (Fig. 2e and f) the nuclear membrane has disappeared. There are, however, around the periphery of the plate delicate, blister-like structures which may be remnants of the nuclear membrane. The chromosomes, which are spherical or ellipsoidal, and perhaps about 300 in number, are arranged on a discoidal plate $13.2\ \mu$ in diameter. The split halves of the chromosomes in some cases can be seen. The spindle fibers are at right angles to the chromosome plate and are about $4\ \mu$ long. No centrioles or granules are apparent at the ends of the spindle fibers. There is still a lighter area in the cytoplasm around the figure.

Anaphase

During anaphase (Fig. 2g) the chromosome plate splits into two daughter plates which diminish in diameter with the chromosomes becoming so closely aggregated that they no longer can be distinguished individually. In late anaphase the plates measure $8.4\ \mu$ in diameter and are, for the most part, flat. A few are slightly arched or saucer-shaped. The spindle between the chromosome groups has in most cases become twisted as if both plates had rotated in opposite directions. The polar fibers seem to be finer and more numerous than the interzonal ones, and the polar areas appear slightly darker than the surrounding cytoplasm. The polar fibers have not shortened during anaphase but are in most cases $5\ \mu$ long, and the outside ones are inclined at an angle of about 60° to the plates.

In an amoeba containing thirty-three nuclei, twenty-nine are in late anaphase and four are in early telophase. The nuclei in anaphase have their polar fibers inclined at an angle of 60° to the chromosome plates. The four in early telophase have granules arranged on a more or less hemispherical surface or membrane (Fig. 2h and i). Twenty-nine of the spindles lie so that the distance between daughter chromosome groups can readily be measured. The average distance between groups is $34\ \mu$. The shortest distance is $10\ \mu$, and the longest distance is $62\ \mu$. Some of the chromosome plates are tilted at angles to each other so that one of a pair is seen in edge view, while the other is seen in polar view. Some pairs of plates, both showing in edge view, are twisted at angles of 30° to 150° to each other. In the twenty-one anaphase spindles which lend themselves to analysis, twenty have the spindle between the chromosome groups twisted clockwise, or to the apparent right (Fig. 2g). One spindle shows no twisting. This constancy in direction indicates that the twisting is caused not by external forces in the cytoplasm, but by forces inherent in the spindle apparatus.

Telophase

In early telophase (Fig. 2h) granules appear at the distal ends of the polar fibers. A delicate, more or less hemispherical, membrane forms at the poles and the ends of the fibers nearest the poles disappear (Fig. 2i).

Later telophase (Fig. 2j) shows the plates larger in diameter, less dense and more finely granular, with the membrane more flattened. The granules are more densely packed on the median sides of the nuclei where the membrane is scarcely visible except near the edge of the disc. The spindle fibers have completely disappeared by this time.

DISCUSSION

Mitosis in *A. carolinensis* as herein described is very similar to that of *A. proteus* as described by Chalkley and Daniel (1933), Chalkley (1936), and Liesche (1938). The figures and descriptions of the nuclear division stages of *A. proteus* agree with those of *A. carolinensis* with the following exceptions:

1. The nuclei and mitotic figures of *A. carolinensis* are approximately half the size of those of *A. proteus*. Also the chromosome number of *A. carolinensis* (probably near 300) seems to be about half that given by Liesche (500-600) for *A. proteus*.
2. No "spireme" (Liesche) stage was observed in *A. carolinensis*.
3. No granules (Chalkley and Daniel) were visible at the distal extremities of the spindle fibers at metaphase.
4. During metaphase and anaphase grouping of the distal ends of the polar spindle fibers is not so pronounced in *A. carolinensis*, and during anaphase the chromosome plates are only slightly arched.

SUMMARY

1. It is concluded that the giant amoeba described by Wilson (1900) is not a *Pelomyxa* but belongs to the genus *Amoeba* and should be designated *A. carolinensis*. *Chaos chaos* is considered to be invalid as a name, owing to the fact that it is based on Roesel's inadequate description.
2. Nutritional studies indicate that *A. carolinensis* has food requirements similar to those of *A. proteus* and that these two species react in a similar manner to the same type of food.
3. Nuclear division is described for *A. carolinensis*. The stages are similar to those described for *A. proteus*, and except for smaller size, correlated with a smaller number of chromosomes, the differences are insignificant.

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THE ELIMINATION OF CHROMOSOMES IN THE MEIOTIC DIVISIONS OF *BRACHYSTETHUS RUBROMACULATUS* DALLAS

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The occurrence of meiotic abnormalities is anything but rare. However, in almost every instance it is the result of mechanical or physiological accidents and therefore sporadic and irregular. Hence, if in a certain species a departure from the normal process always takes a very particular form and is restricted to a certain region of the gonad, it is likely that it is not entirely accidental. If, furthermore, every male or female in the species is affected, it is safe to assume that we are dealing with a basic and well regulated condition, unorthodox though it may be.

The case in question is that of *Brachystethus rubromaculatus*, one of the pentatomid Hemiptera. It holds for all the males of the species. The testis here is composed of four lobes and in the fourth of these (counting from the side where the sperm duct makes its exit) the meiosis follows an abnormal but very definite course. In a way, this is analogous to the case of *Loxa* (Schrader, 1945a and b), but that is only in the sense that in both the exceptional development takes place in a certain lobe of every testis. The nature of the abnormality is quite different in the two species, as will appear in the account below. My chief interest lies in the possibility that such irregularities may be of use in the analysis of the ordinary mechanism of mitosis and in this as well as several succeeding studies I hope to show that they may throw some light on certain puzzling aspects of the division cycle.

MATERIAL AND METHODS

The gonads of four males and one female were used in the investigation. The insects were collected near Turrialba, Costa Rica, during April and May, 1944. The material was fixed in Sanfelice and sectioned at 5 to 10 μ . Gentian violet, haematoxylin and the Feulgen reaction were used in staining, the haematoxylin being especially useful in the analysis of spindle conditions whereas the Feulgen reaction is indispensable in following the maneuvers of the chromosomes. The main manifestations of the mitosis are so clear that they are well shown by pen and ink drawings—a method which in most other cases does not give a just presentation of spindle conditions.

I take pleasure in expressing my gratitude to Dr. E. N. Bressman and Mr. R. A. Nichols of the Inter-American Institute of Agricultural Sciences for facilitating the work. My special thanks are due to Dr. T. J. Grant of the U. S. Department of Agriculture, whose ready helpfulness did so much to further my researches in Costa Rica.

NORMAL SPERMATOGENESIS

The testis has only four lobes of which the first three show an orthodox spermatogenesis differing in no essential from that described in so many other pentatomids.

The fourth or "harlequin" lobe has about the same proportions as the rest and does not differ in any discernible way in its general organization.

The size differences among the chromosomes as seen in the spermatogonia are not very great. But it is possible to recognize one large, four medium and one small pair of autosomes. The X is a trifle larger than one of the small autosomes while the Y is the smallest chromosome of the complement (Fig. 1). Identification of the sex chromosomes was checked by examination of oögonial metaphases in the female.

The normal meiosis shows the usual prophase conditions, with the leptotene, synaptotene, pachytene, and diplotene stages followed by the confused period in which chromosomal behavior is difficult to analyze. This is succeeded by a diakinesis marked by the appearance of beautiful tetrads which, as they condense, are distributed around the nuclear periphery. Just before the breakdown of the nuclear membrane this rather even distribution begins to disappear and the chromosomes may even come in contact with each other in small groups of two or three each (Fig. 2). This collocation reaches its height when the membrane disintegrates. All of the chromosomes then huddle together in the middle of the nuclear space only to separate again almost at once to form the equatorial plate. In the latter they follow the characteristic pentatomid arrangement of the first metaphase, with one or both sex chromosomes taking a central position within a ring of autosomal tetrads (Fig. 3).

The sex chromosomes are marked by their heteropycnosis from the leptotene period on. Until the synaptic period they are well separated from each other, but then tend to come together. During the confused period they form a single, rounded chromatin nucleolus and it is not until after the early diakinesis that they once more become independent of each other. They divide equationally in the first, and segregate in the second division, so that the spermatids carry $6 + X$ or $6 + Y$ chromosomes (Figs. 3-5).

There is no interkinesis and the two centrioles at each pole have already separated to assume their position for the second division before the chromosomes have completed the anaphase movement of the first. This precocious behavior of the centrioles is however also encountered in several other pentatomids and does not interfere with the normal meiotic distribution of the chromosomes except in such extreme cases as *Peromatus* (Schrader, 1941b).

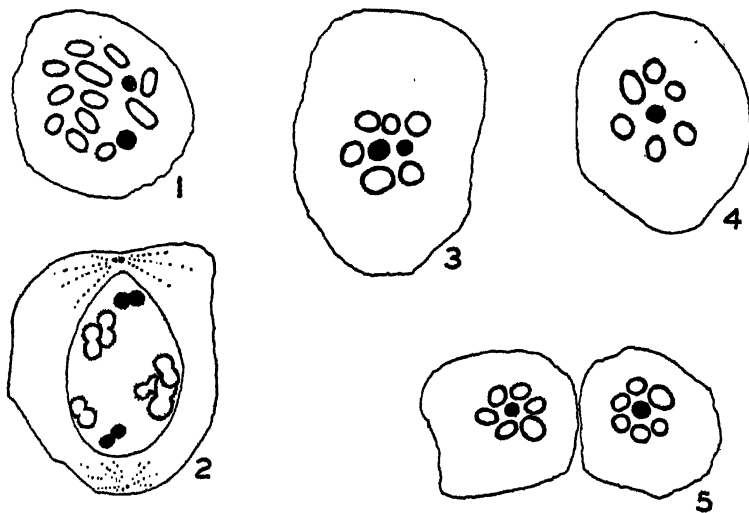
ABNORMAL SPERMATOGENESIS

First division

Neither in the spermatogonial nor the meiotic behavior up to the late diakinesis does the harlequin lobe differ from the other three. Six autosomal tetrads are formed which resemble in every way those seen in the normal lobes (Fig. 6). Again, as these tetrads condense into the characteristic dumbbell shaped bodies, they and the sex chromosomes are well distributed around the nuclear periphery.

But then arises a difference in behavior from the normal. Here, too, the last phase prior to membrane disintegration witnesses a coming together of the chromosomes. In this fourth lobe, however, the collocation is both more regular and more pronounced. In nearly all cells the six tetrads form a single chain whose constituents maintain contact with the nuclear periphery. This chain is always more

or less equatorially placed (Fig. 7 and 8). The configuration is evidently the resultant of several different forces. Just as in normal cells, the tendency to collocate begins to manifest itself in late diakinesis. But in this fourth lobe the autosomal tetrads are also repelled by the two opposite poles at this early stage. Since the chromosomes are still confined within the nuclear membrane they consequently move into the middle region. The combination of polar repulsion, the tendency to collocate, and adhesion to the still intact nuclear membrane must perforce result in the formation of an equatorial chain.



Drawings were made from haematoxylin preparations, except for Figures 12 and 24. All figures magnified approximately 1400 \times . Autosomes drawn in outline, and sex chromosomes in solid black throughout.

FIGURE A. Normal spermatogenesis. 1. Spermatogonial metaphase; 12 autosomes and X (large) and Y. 2. Late diakinesis; beginning of autosomal clumping. 3. Metaphase of Division I; X and Y in middle. 4. Metaphase of Division II; X superimposed on Y. 5. Telophases of Division II showing two types of spermatids: 6 autosomes + Y, 6 autosomes + X.

As one might expect, there is no constant seriation in such a chain. When the nuclear membrane disintegrates, the chain is converted into an irregular clump just like the clump formed by the tetrads of normal cells that have undergone no such maneuvers (Fig. 9 and 10). Much less frequently is there a formation of two smaller aggregates instead of the single large one. The two sex chromosomes are included in such groupings only by accident and sooner or later they always separate from the autosomes and assume an independent position. This is not necessarily an equatorial one at first (Fig. 8-10).

It is, however, in the establishment of the first metaphase that the most striking departures from an orthodox behavior are evinced. As already stated, the breakdown of the nuclear membrane is followed immediately by the clumping of the autosomal tetrads in the midregion. In many cells this may, however, be halted temporarily if a central core of spindle fibers is formed quickly between the poles. The clumping autosomes may then be applied to these central fibers in a half moon

configuration for a moment (Fig. 11). But in any case, before the metaphase spindle has assumed final shape, the autosomal aggregate is shifted out of this general middle region. Almost always this movement seems to be a sudden one and frequently the aggregate comes to rest rather close to the lateral wall of the cell, the direction of the shift being toward the side and never toward the poles. The cell frequently bulges out on the side on which the aggregate is located (Fig. 14). If instead of one aggregate, two smaller ones have been formed, they undergo similar reactions and often become displaced toward opposite sides of the cell (Fig. 13).

The beginning of this autosomal shift sees the X and Y in no definite position although usually in the vicinity of the polar axis. Frequently they are situated at some distance from the equatorial plane and bear neither a constant positional relation to each other nor a mitotic orientation toward the centrioles (Fig. 9 and 10).

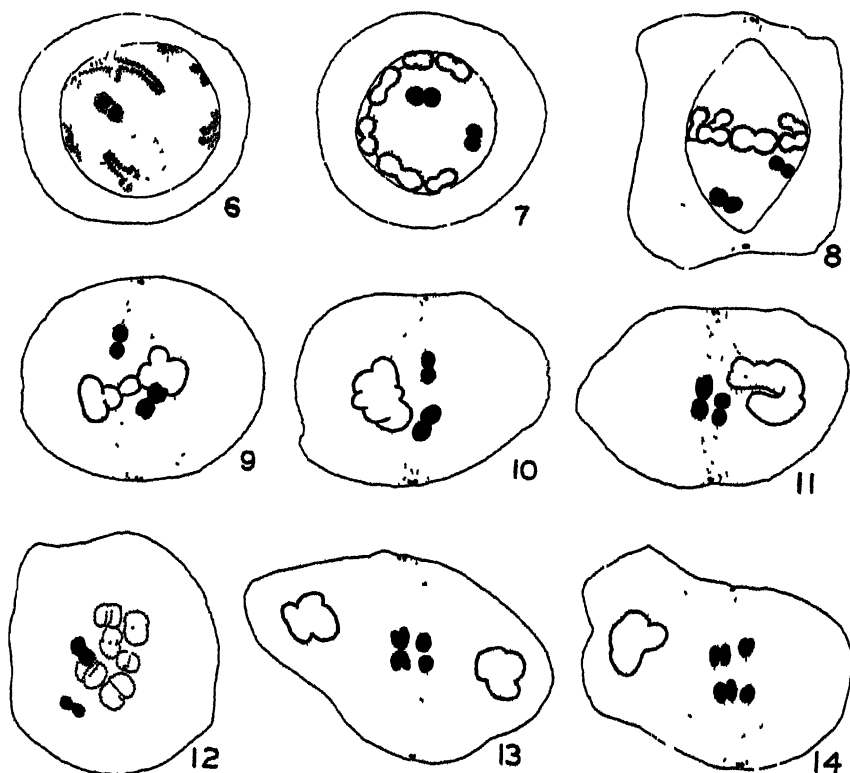


FIGURE B. Abnormal spermatogenesis. Division I from diakinesis to early anaphase. 6. Mid diakinesis showing tetrads of normal appearance. 7. Late diakinesis in polar view; autosomal tetrads forming equatorial chain. 8. Late diakinesis in side view. 9. Early stage in clumping of autosomal tetrads. 10. Beginning of movement of autosomal aggregate away from polar axis. 11. Displaced autosomal aggregate applied to central portion of spindle in half moon form; X and Y assuming equatorial position. 12. Feulgen preparation corresponding to Figures 9 or 10 showing that tetrads retain their individuality in the aggregate. 13. Autosomes in two aggregates, both shunted out of polar axis. 14. Early anaphase, with X clearly showing "tertiary" split; characteristic displacement of autosomal aggregate.

However this situation is quickly altered and as the autosomal aggregate moves toward the side of the cell, the X and Y approach very close to the polar axis and assume a position in the equator with a definite orientation toward the poles (Fig. 11 and 13).

In all of the hundred or more cells observed at this stage, such a configuration of autosomes and sex chromosomes is always maintained. Since in the prometaphase the two types of chromosomes form one general group albeit not always in contact with each other, it is clear that the later separation is not an accidental one. The unusual shift of the autosomes is equivalent to an actual extrusion from the mid-region of the cell.

Despite their distance from the polar axis, the autosomes continue to be connected with both poles by chromosomal spindle fibers. This is at first glance rather surprising since with ordinary stains like gentian violet and haematoxylin the autosomal aggregate appears as a solid, structureless mass which plainly suggests degeneration. In good Feulgen preparations, however, it becomes clear that the autosomal tetrads have by no means lost their individuality. All six of them can be easily distinguished, lying in a substance which does not stain with Feulgen and thus reveals the individual chromosomes (Fig. 12). This substance, which with other stains becomes just as dark as the chromosomes themselves, resembles the material surrounding the sex chromosomes of certain reduviid Hemiptera (Troedson, 1944) and may also be akin to the "flocculent, whey-like coagulum" which envelops some regions of the chromosomes in *Olfersia* (Cooper, 1944). In *Brachystethus* it is evidently formed when the chromosomes clump at prometaphase and it persists through both meiotic divisions.

In the middle of the cell, between the two poles, there is a well-formed spindle of normal length, which however is smaller in diameter than the spindle of normal cells. This accommodates the two sex chromosomes, which are located in the middle of the spindle substance just as they would be if the autosomes were free to form a ring around them.

Despite the presence of the chromosomal fibers which connect the autosomes with the poles, the autosomal aggregate behaves more or less like an inert mass in the mitosis that follows. While the sex chromosomes undergo an equational division and approach the poles, the autosomes near the periphery of the cell undergo no movement (Fig. 14-17). It is only when the dividing cell elongates and becomes narrow that the autosomal clump is moved toward the midline (Fig. 18). If it there comes in contact with the expanding interzonal region or "Stemmkörper" it may be swept along into one of the two daughter cells, but very often it is not until the cleavage constriction is being completed that the aggregate is definitely included in either of the resulting cells (Fig. 19 and 20). Apparently the chromosomal fibers exert little or no traction at this time. So far as one can tell, the inclusion of the autosomes in either daughter cell occurs entirely at random.

It is of interest to note that although in its essentials the equational division of the sex chromosomes is always accomplished successfully, there are certain features that distinguish it from the corresponding process in normal cells. In the first place, the sex chromosomes and especially the X clearly show a tertiary split already at this first metaphase—a split which is not utilized until the first somatic division in the egg (Fig. 13-15). This is sometimes indicated in normal cells also, but never so strikingly as here where it appears after all three of the stains used. The second

feature lies in the anaphase behavior of the sex chromosomes. Although both start out from an equatorial position, the early anaphase often shows the chromatids of the Y much closer to the poles than those of the X (Fig. 15). In other words, the Y precedes the X in the poleward movement. When there is such a disparity of movement, there is frequently a shift during mid anaphase through which these four chromatids are all brought into the exact polar axis in a single line. Since as just stated, the chromatids of the Y move more quickly, they constitute the extremities in this tandem arrangement, whereas the two X chromatids are in between (Fig. 16). Such a seriation strongly resembles that which characterizes the second di-

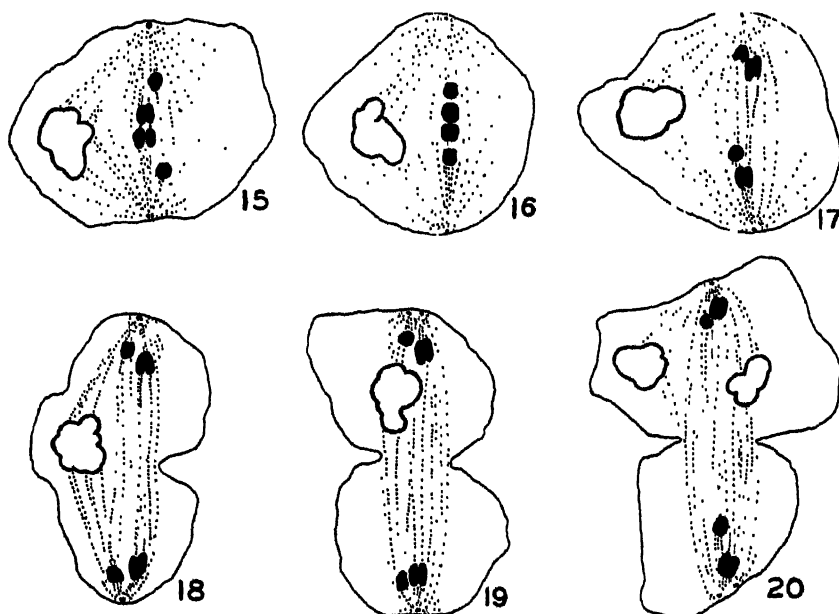


FIGURE C. Abnormal spermatogenesis. Division I, anaphase and telophase. 15. Early anaphase with Y preceding to the poles. 16. Early anaphase; X chromatids have moved into polar axis between separating Y chromatids. 17. Mid anaphase. 18. Late anaphase; autosomal aggregate returning to axial region. 19. Early telophase; an X and a Y at each pole with the autosomal aggregate included in upper cell. 20. Late telophase with two autosomal aggregates both included in upper cell.

vision of the coccid *Protortonia* (Schrader, 1931), and there is little doubt that similar forces are involved. This anaphasic shift does not affect the result of the division; one X and one Y chromatid go into each of the resulting second spermatocytes.

Thus in the majority of cases, two types of second spermatocytes are produced: one carries an X and a Y, as well as the clumped autosomes; the other contains only the two sex chromosomes (Fig. 19). If the autosomes have been aggregated in two masses before metaphase, these may both go to the same pole or to opposite poles, apparently at random (Fig. 20). In no case is there a division of the individual tetrads.

Second division

As in normal cells, the centriole at each pole of the first spindle is divided and the two daughter centrioles separate some time before the division has been finished (Fig. 21). Each moves through an arc of 90° and the new polar axis for the second division is therefore at right angles to the first. Even while still in telophase, the two sex chromosomes often respond to the new poles and move toward them (Fig. 21). However, this precocious movement is soon reversed and the X and Y then come together in the middle of the new spindle in their orthodox "touch and go" pairing (Fig. 22 and 28) and it is only following this that they separate in the regular segregation toward opposite poles. There is no indication of any interkinesis.

The autosomal aggregate may at first remain in the general region where it has entered the cell upon completion of the first division, but in other cases it approaches closely to the two sex chromosomes that are near the new polar axis (Fig. 21). Such an approach is only temporary, however, and before the second division is initiated the aggregate is always extruded from this middle region just as it was in the first division (Fig. 23). The configuration of the second division thus almost duplicates that of the first.

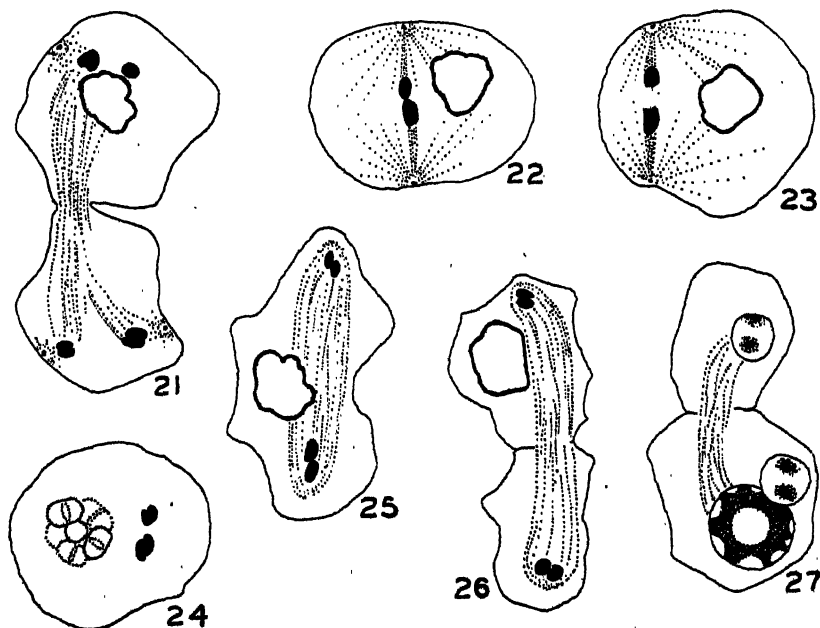


FIGURE D. Abnormal spermatogenesis. Division II of cell containing autosomal aggregate (all drawings except Fig. 21 show X below Y). 21. Beginning of Division II; X and Y reacting to new position of poles. 22. Touch and go pairing of X and Y; autosomal aggregate shunted away from polar axis. 23. Early anaphase; X and Y going to opposite poles; autosomal aggregate in characteristic displacement. 24. Feulgen preparation showing that tetrads still appear unaltered in early phase of Division II. 25. Anaphase; autosomal aggregate returning toward polar axis. 26. Telophase; autosomal aggregate included in cell with Y. 27. Spermatids; upper cell carries only a micronucleus with the Y half chromatids; lower cell with micronucleus containing X half chromatids and autosomal nucleus.

The autosomal tetrads are still intact and are still imbedded in the substance that stains intensely with gentian violet and haematoxylin. However, the aggregate is usually smaller than it was just prior to the first division, a fact that results from a greater crowding of the tetrads, as can be seen in Feulgen preparations (compare Fig. 24 with Fig. 12). Again, chromosomal fibers are formed (Fig. 22), although the aggregate seems to be moved about as a more or less passive body (Fig. 25 and 26). In short, just as in the first division it is only the sex chromosomes that are involved in the regular mitotic mechanism. But now, as in normal second divisions, the process is reductional and the resulting cells receive only an X or a Y (Fig. 26 and 30). The autosomes if present are once more included in either cell as a group, and so far as one can tell, on the basis of chance (Fig. 23-26).

The spermatids that result from these two peculiar divisions thus either carry only one sex chromosome (the X or the Y) or else they contain the autosomal aggregate in addition. In other words, there are four main types of spermatids: X; Y; X + autosomes; Y + autosomes (Fig. 26 and 30).

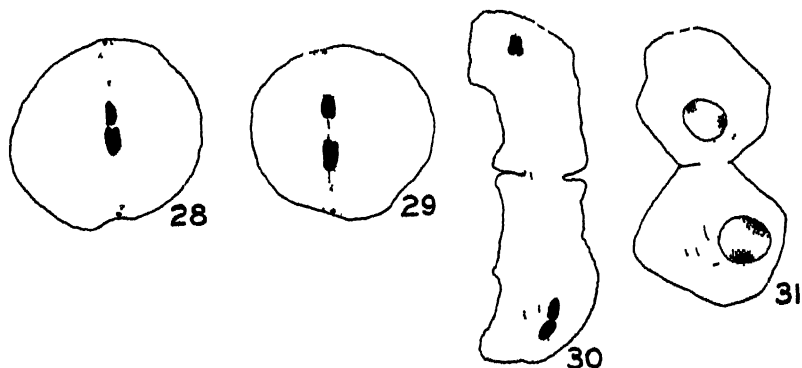


FIGURE E. Abnormal spermatogenesis. Division II of cell lacking autosomes (all drawings show X below Y). 28. Touch and go pairing of X and Y. 29. Early anaphase. 30. Late telophase. 31. Spermatids; upper cell with micronucleus containing the two Y half chromatids, lower with two X half chromatids.

If the autosomes are gathered in two instead of the usual single group prior to the first division, the resulting spermatids may of course carry intermediate numbers of chromosomes. But for the great majority of cells they are transmitted in a single aggregate. Throughout there is no attempt at a division of the individual autosomal tetrad, and it is as an aggregate of tetrads that the autosomes enter the spermatid. Obviously there is some factor which interferes with their mitotic mechanism—a factor which interferes in no way with that of the sex chromosomes. The latter behave just as they do in the normal cells of neighboring lobes of the testis.

Spermateleosis

The first steps after the telophase of the second division parallel the normal course of events. The transformation of the autosomal mass into the spherical nuclear structure shown in Figure 27 is peculiar, though in later stages it approaches

closely the condition seen in the normal spermatid. In these abnormal cells however, the sex chromosome at first forms a micronucleus which is distinct from the larger autosomal nucleus (Fig. 27). But sooner or later, the micronucleus becomes applied to the large nucleus and gradually merges with it.

When, as in most cases, a single sex chromosome constitutes the only chromosomal constituent of the spermatid, its behavior differs in no way from that in cells containing the autosomes as well. In either case a tiny nucleus is formed by each sex chromosome and in this the constituent half chromatids separate to form two distinct chromosomal bodies. (Fig. 27 and 31). This precocious separation is not surprising when it is remembered that already in the first metaphase these half chromatids can be clearly identified as such.

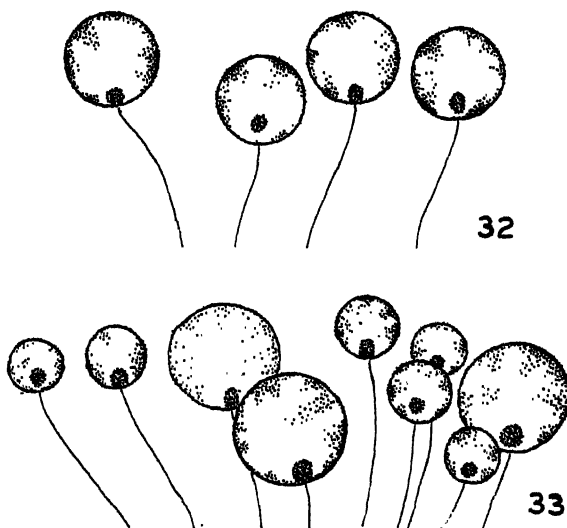


FIGURE F. Spermatids before elongation. 32. Normal spermatids shown to indicate size. 33. Abnormal spermatids at stage corresponding to Figure 32. Largest spermatids contain all autosomes as well as either X or Y. Smaller spermatids carry either X or Y; latter is the smallest.

The process of transformation into the sperm seems at first alike in all spermatids and homologous to that observed in the normal lobes. This is true whether they carry only a single chromosome or the autosomes as well. As might be expected, the former are distinctly smaller than the corresponding normal cells and nuclei (Fig. 32 and 33); the latter on the other hand are markedly larger. It is also interesting to note that it is often possible to distinguish between the small sperms carrying either an X or a Y. The latter are the smaller of the two (Fig. 33). However, when the rounded sperm heads begin to elongate, those which carry only a sex chromosome begin to stain less intensely than either normal or "autosomal" abnormal sperms, and as the elongation into the typical tenuous sperm head continues it becomes more and more difficult to trace their further fate even in Feulgen preparations. It is likely that the "sex chromosome sperms" never attain

the final stages of sperm formation. However, this cannot be made certain without smear preparations for even in very thick sections the long sperm heads are practically always cut and it is impossible to decide whether one is dealing with an abnormally small sperm or only a portion of a larger sperm. The fate of the giant sperms, however, is subject to no such doubt. They continue their development to the fully formed stage, enter the sperm duct, and are there mingled with the normal sperms from the normal lobes.

GENERAL CONSIDERATIONS

The cytological features that characterize this abnormal development are thus of the most striking sort. The most obvious one lies in the clumping of the autosomes. A temporary collocation of chromosomes is of course encountered as a normal occurrence in many species, but is there, confined—as it is in the normal lobes of *Brachystethus*—to a very brief, almost momentary period immediately after the breakdown of the nuclear membrane in prometaphase. In *Brachystethus* there is some tendency toward a collocation of autosomes even before this event. But in the abnormal lobe, the clump that is finally formed is not resolved again, and this condition is maintained through both meiotic divisions and into sperm formation. Moreover this collocation of chromosomes is so intimate that only a Feulgen preparation reveals that there has not been an actual fusion.

Although the further behavior of this autosomal aggregate suggests strongly that it is shunted about more or less like a passive body during cell division, it nevertheless evinces certain reactions which indicate that it is not completely inert. In diakinesis, these autosomal tetrads duplicate the behavior of normal tetrads in establishing contact with the nuclear periphery; toward the end of the diakinetid period they evince a reaction to the two poles in taking up an equatorial position in contact with the nuclear membrane; after the disappearance of the latter they form chromosomal fibers to the poles; and almost simultaneously they move out of the midregion of the cell with a suddenness that bespeaks a forcible displacement.

It is possible and even likely that this movement toward the side of the cell is due to the same centriolar repulsion which forces the chromosomes into the equatorial plane while they are still held within the confines of the nuclear membrane. An influence of centrioles on the chromosomes prior to the dissolution of the nuclear membrane is observed in certain other forms also (for instance in *Anisobalis*, Schrader, 1941a). But not often is it exerted so as to bring about an equatorial placement at so early a stage, even though a role in the later final formation of the equatorial plate is frequently assigned to it.

It will be realized, however, that even in normal cases, additional factors must function to restrict the chromosomes to the middle of the equatorial plane. Repulsion from the two poles alone cannot do that. It is possible that the chromosomes are thus confined to the midregion simply because of surface tension conditions that prevail in the spindle body at the time of metaphase. The escape of the autosomes in the present case might then be attributed to abnormalities in the spindle, say to untoward alterations in the timing of the normal viscosity changes. But that is clearly not the entire explanation since the autosomes are not accompanied in their displacement by the two sex chromosomes. The latter remain in the spindle in a quite orthodox position. Since the cytoplasm, the centriolar forces,

and the general spindle conditions are identical for both autosomes and sex chromosomes, it is therefore in the chromosomes themselves that the explanation must be sought. More specifically, the question to be solved is why the autosomes do not respond to the forces which at metaphase counteract the influence of the centers and confine the chromosomes to the middle of the equatorial plane.

The abnormal condition of the autosomes is not indicated by any striking behavior during the prophase. There is only a more pronounced tendency to assume an equatorial position at prometaphase and the formation of chains that are absent in normal cells. Also, after the aggregate has been formed, Feulgen preparations show the component tetrads to be somewhat swollen and less intensely stained than are the prometaphase tetrads. This probably indicates the first step in a return to a diffuse state. Such a regressive condition may in some way be responsible for the special maneuvers of the autosomes, for the X and Y chromosomes which during this time remain fully condensed behave just like the sex chromosomes of normal cells. But it is not certain that this would suffice as an explanation since in addition to the clumping of the autosomes one must also account for their elimination from the midregion and their failure to take the first steps in division. For it must be remembered that the initial separation of daughter chromosomes is an autonomous process which is independent of spindle action. Nevertheless, although the *Brachystethus* tetrads appear ready for such a step and the line of demarcation between the paired chromosomes is clearly marked and complete, no separation ever occurs. Either there is no mutual repulsion or else the pellicle and the achromatic constituents of the tetrads prevent the normal division. In any case it is clear that some of the basic reactions of the chromosomes have been altered.

It is natural to seek a parallel in other cases already on record. The investigation of certain echinoderm hybrids by Baltzer (1910) is especially pertinent. Baltzer found that in certain of these crosses, the paternal chromosomes are eliminated during cleavage. There, too, is a tendency for such chromosomes to clump, and there also a formation of chromosomal spindle fibers occurs nevertheless. Likewise there is sometimes a lateral elimination of these chromosomes although they usually simply lag in the middle of the spindle. However, all this does not occur until the anaphase is well advanced and the various configurations are by no means as constant as they are in *Brachystethus*. After a careful analysis, Baltzer concluded that it is the chromosomes themselves rather than the plasma that is responsible for the elimination. That such a conclusion is warranted for the case of *Brachystethus*, as well, has already been pointed out. The difference in behavior between the autosomes and the sex chromosomes in the same plasma makes it unavoidable.

The elimination of chromosomes consequent on a loss of their kinetochores is observed regularly in certain molluscs (Pollister, and Pollister, 1943). Such an explanation is worthy of serious consideration in the case of *Brachystethus*. However, there can be only a partial loss of the kinetochore activity because some chromosomal fibers are evidently still being formed. Since we are dealing here with a diffuse kinetochore, such a partial loss is easily conceivable, but it must be confessed that the akinetic chromosomes of molluscs present very different elimination pictures than those seen in *Brachystethus*.

The conditions in such coccids as *Phenacoccus* (Hughes-Schrader, 1935) may also be germane to the present case. There, too, certain chromosomes betray a

tendency to clump, and it is these chromosomes that become inert and degenerate within one or two succeeding divisions. Nevertheless, these chromosomes form normal chromosomal fibers.

But all these other instances are themselves in need of further explanation. The only conclusion that protrudes itself in such an analysis is that the abnormality is to be sought in the basic organization of these forms and is not a superficial and accidental one. The alterations that are involved primarily affect the chromosomes and influence their reactions to each other and to the mitotic mechanism.

EVOLUTIONARY ASPECTS

It is very questionable whether spermatids carrying only an X or a Y chromosome ever develop into mature sperms. That, however, is not true of the giant sperms which in addition to a sex chromosome carry the full set of autosomes unaffected by any meiotic mitosis (i.e., four times the number of autosomes contained in a normal sperm). But though these large sperms enter the sperm duct and mingle with the normal sperms, it is doubtful whether they ever become functional in the hereditary sense. Of the seven specimens of *Brachystethus* that I have examined, none was marked by unusual morphological features such as confidently might be expected if a sperm nucleus with a quadruple set of autosomes joins the haploid nucleus of a normal egg.

But if these numerous giant sperms play no direct role in the hereditary mechanism of the species, it becomes a matter of wonder that such an extensive development of abnormal gametes could have withstood the effects of natural selection. For it must be remembered that the testis of *Brachystethus* has only four lobes and if the sperms of one of these do not function in the genetic determination of the embryo, we are confronted with a prodigious waste which is added to that which occurs normally in the reproduction of most male animals.

Such a waste is paralleled in several species of *Loxa* (Schrader, 1945a and b) where there is likewise an abnormal lobe in every testis. Although the nature of the aberrancy is different in the two cases, they are similar in that both encompass the production of a huge number of sperms which carry many times the normal number of chromosomes. It seems strange that two processes so diverse in their abnormalities should culminate in gametes whose general characters are so similar.

The explanation may lie in the fact that though these large sperms take no part in the direct control of the heredity of the species, they may nevertheless be important in its welfare. Since they are normal in every respect except size, it is more than likely that they enter the egg like the normal sperms. It also must be recalled that polyspermy is almost universal in the fertilization of insect eggs and that in many species the number of supernumerary sperms is very high. The entrance of genetically inert sperms would probably offer no difficulties in the regular fertilization process and a union between two normal pronuclei would proceed as usual.

It is clear from evidence in marine eggs that the breakdown of gamete nuclei releases substances which have a far reaching influence on the reactions in the egg. It is likely that such substances are present in abnormally large quantities in the giant sperms and that therefore their possibly beneficial effects are increased. Again, if the developing embryo utilizes the nucleo-proteins that are brought in by

supernumerary sperms, an advantage might well accrue to a species which has such substances available in the unusually large amounts that are represented in the giant sperms. In short, the latter may play a not inconsiderable role in the embryology.

If that is the case, the harlequin lobe would in no sense be a useless organ. The development of such a lobe in the testes of two genera that are taxonomically as diverse as *Brachystethus* and *Loxa* may well have been due to a nongenetic but metabolic advantage that is thereby conferred on the developing embryos.

SUMMARY

1. The fourth lobe in all testes of the pentatomid *Brachystethus rubromaculatus* shows an aberrant meiosis of a very definite character.

2. The first indication of this aberrancy is found in a chain formation and clumping of the autosomal tetrads just prior to metaphase. The two sex chromosomes are not included in this aggregation of autosomes.

3. At metaphase the X and the Y take a normal position in the spindle but the aggregate of autosomes is shunted laterally out of the middle region of the cell, away from the polar axis.

4. The two sex chromosomes divide equationally in the first division while the autosomal aggregate passes unaltered to either pole.

5. In the second division the X and Y again behave as in a normal meiosis and separate to opposite poles. The autosomal aggregate, if present, passes to either pole apparently at random.

6. Since the clumped autosomal tetrads pass undivided through both divisions while the sex chromosomes behave normally, the following four main types of spermatids are produced: X; Y; X + autosomes; Y + autosomes.

7. The mechanics and evolution of so constant an aberrancy are discussed.

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AN INVESTIGATION OF CROSS STRIATIONS AND MYOSIN FILAMENTS IN MUSCLE *

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INTRODUCTION

Present information on muscle structure is of two rather distinct types, that derived from histological studies through direct observation with the light microscope, and that deduced from indirect methods such as involve polarized light microscopy, streaming double refraction of myosin solutions or x-ray diffraction. Histological studies have always been limited by the inadequate resolving power of light microscopes; the indirect methods have been limited by the fact that they can supply information only in proportion to the correctness of the necessary assumptions. Although valuable knowledge has resulted from both types of approach, there is a lack of definite information on structures below the limit of light microscope resolution. The electron microscope provides the possibility of extending direct observation into a range of dimensions reached hitherto only by indirect methods and conjecture. Since success in biological electron microscopy depends to a large extent on the development of techniques suitable for the material, these initial observations may be expanded as new techniques are developed.

It is impossible in the present space to summarize the previous research on muscle or the ideas put forth concerning its structure; reference to the literature will be limited to those papers which provide a basis for the present study. A general review has been presented by Fenn (1945) while the histological aspects have been reviewed by Jordan (1933). Polarized light investigations have been reviewed by Schmidt (1937) and by Weber (1934). Results of investigations of streaming double refraction in myosin solutions may be found in the papers of Edsall (1930, 1942), von Muralt and Edsall (1930), Mehl (1938) and Dainty, Kleinzeller, Lawrence, Miall, Needham, Needham and Shen (1944). Astbury and Dickinson (1940) and Astbury (1942) described wide-angle x-ray diagrams of muscle and myosin, and Bear (1944, 1945) described small-angle x-ray patterns of various muscles. A discussion of muscle physics has been presented in a review by Ramsey (1944).

In the past, the electron microscope has been used very little in the study of muscle structure. Richards, Anderson, and Hance (1942) have shown electron micrographs of a wedge-cut section of cockroach striated muscle in which each darker band can be seen to consist of three components. Sections of muscle tissue cut by another method were examined by Sjöstrand (1943) but the electron micrographs bear no obvious relation to known structures in muscle. In both these stud-

*The substance of this paper was presented at the meetings of the Electron Microscope Society of America on December 1, 1945.

ies the object was primarily to investigate sectioning techniques for electron microscopy rather than to study muscle itself.

Ardenne and Weber (1941) published electron micrographs of filamentous myosin particles, which are undoubtedly to be identified with the asymmetrical particles responsible for the streaming birefringence phenomena in myosin sols. Hall, Jakus, and Schmitt (1945) have studied another fibrous protein from molluscan muscles and correlated the structure with the x-ray diffraction results described by Bear (1944).

ELECTRON MICROSCOPE OBSERVATIONS OF MYOFIBRILS

Preparation of specimens

A primary difficulty in the study of striated muscle with the electron microscope is the preparation of the tissue thin enough to be partially transparent to the electron beam. In view of the difficulties connected with present microtome techniques, a preparative method was sought in another direction. One procedure which has been used previously depends on the tendency of many materials to fragment along natural cleavage boundaries. If such tendency to natural cleavage exists, it may be assisted by chemical or physical means or both. Thus collagenous tissue may be separated into fine fibrils by mechanical "teasing" and the separation is facilitated by weak acid (Schmitt, Hall, and Jakus, 1942). Such methods of fragmentation are applicable to many materials and are readily adapted to striated muscle.

Fresh muscle is fixed in 10 per cent formalin, cut into small pieces and subjected to mechanical agitation in a Waring Blendor. When the resulting suspension is lightly centrifuged to throw down the larger fragments, the supernatant exhibits the characteristic sheen associated with fibrous suspensions. It consists mostly of thin fibrils which may be washed and applied directly to a conventional electron microscope specimen grid with collodion membrane. These fibrils are to be identified with the myofibrils, or sarcostyles, of the muscle fiber.

Most of the observations to be described were made on fibrils from the leg muscles of frog and rabbit. The muscles of lobster and scallop (striated portion) were found to contain relatively large fibrils which were usually quite opaque in the electron microscope. It was necessary to apply fixatives to all these muscles in order to obtain intact fibrils. Wing muscles of the fly, on the other hand, could be teased apart in weak saline solution without previous fixation.

Most myofibrils from frog muscle have widths between 0.5 and 1.0 μ , although a few may be found as wide as 3.0 μ or as narrow as 0.2 μ . They appear to be ribbon-shaped on the electron microscope specimen holder but it is not possible to say whether this shape results from forces produced during drying or whether it represents the form of the myofibril in the intact tissue.

Although most of the structural features of the myofibril can be observed without the use of stain, contrast in the image may be increased by the application of phosphotungstic acid to the specimen (0.1 per cent solution at pH 3-5 for about one minute). The staining procedure is found to be particularly useful in increasing the contrast of the myosin filaments.

An RCA electron microscope Type B with accelerating voltage raised to 65 KV was used throughout the study. A higher voltage would probably be advantageous for this type of material but is not readily obtainable from the standard power supply.

Unstained fibrils

Unstained myofibrils from striated muscle are characterized by a succession of transverse bands of varying density, the main features of which can readily be identified with the bands previously described in histological studies (Jordan, 1933). An electron micrograph of a typical fibril from a slightly stretched frog sartorius is shown in Figure 1, together with the histological designations. The repeating unit, or sarcomere, is bounded terminally by a narrow dense band usually referred to as the *Z* membrane or telophragma. This band is in the center of a light region, the *I* (or *J*) band, so called because it is relatively isotropic. Contiguous with *I* is the sharply-defined *A* (or *Q*) band which is optically anisotropic and has a higher scattering power for the electron beam than does *I*. In some myofibrils the *H* disc (or median disc of Hensen) appears as a lighter region in the middle of the *A* band (Fig. 1). Bisecting the *A*, or the *H* disc if it is present, is the narrow dark *M* band (or mesophragma). There may also appear, in either half of the *I* band, a relatively dark band designated as *N* in Figure 2. These bands are not always present and have been considered by some cytologists to be artifacts resulting from the lining up of granules. In the present study they have been noted in rabbit muscles but are either faint or absent in frog muscles. The clarity of their appearance in rabbit fibrils leaves no doubt as to their existence in this formalin-fixed material.

Myofibrils from rabbit muscle usually show two to four fine cross bands symmetrically disposed with respect to the *M* band and near it. They are beyond light microscope resolution, being about $0.1\ \mu$ from center to center, and represent a periodic variation in the dense material of the *A* band.

In favorable cases fibrils can be seen to consist of longitudinal myosin filaments which are difficult to resolve in unstained fibrils except where they fray out at the edges. They are visible in various parts of Figures 1 and 2.

Stained fibrils

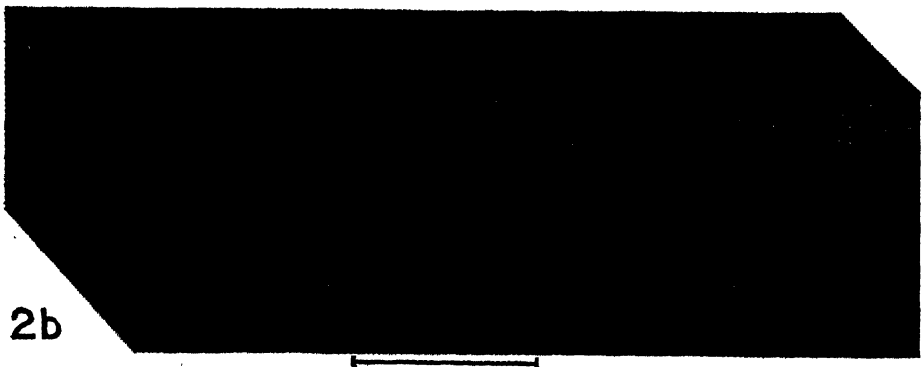
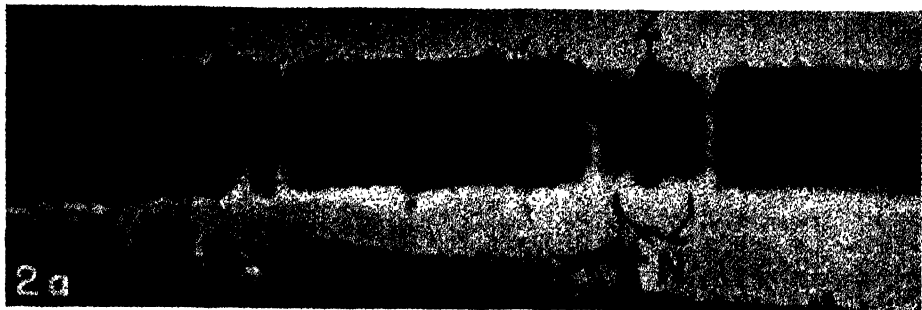
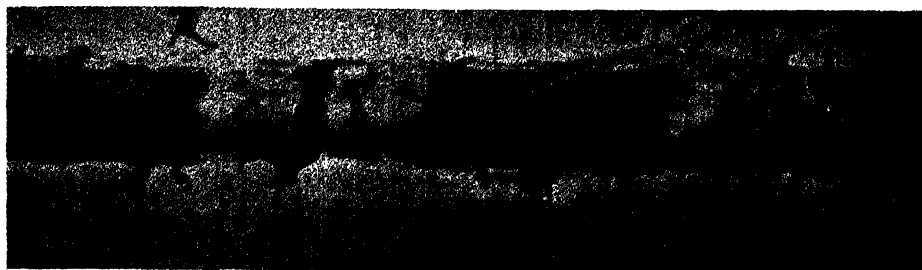
Phosphotungstic acid combines with the cross bands roughly in proportion to their intrinsic density and serves to increase the contrast of the structures described above. Segments of stretched myofibrils stained with phosphotungstic acid are shown in Figure 3. The *A* band is stained more heavily than is the *I* band, and the *M* and *Z* bands appear quite opaque. When the *H* disc is present, it absorbs less stain than does the remainder of *A*.

Besides accentuating the transverse bands, phosphotungstic acid enhances the contrast of the longitudinal myosin filaments. These range in width from 50 to 250 Å and extend continuously, and in relatively straight lines, through both *A* and *I* bands. Although the filaments are usually indistinguishable within the dense *Z* bands, they can be traced through several successive sarcomeres when the *Z* bands are partially disintegrated. In the *A* band the filaments are relatively dense, sharply defined, and almost parallel in orientation. In the *I* band they are less perfectly aligned and of lower density. The higher density of the *A* band appears to result from the higher density of the component filaments.

FIGURE 1. Myofibrils from frog sartorius, stretched about 30 per cent, unstained. $\times 25,000$. All myofibrils shown in this and subsequent figures were fixed in 10 per cent formalin.

FIGURE 2. Myofibrils from rabbit leg muscle, unstained. $\times 25,000$.

PLATE I



In both bands, the myosin filaments present a knotted or beaded appearance with frequent constrictions and variations in density. The nodes are often almost equidistant along the filament and, in some regions of the fibril, they may be aligned laterally to produce a fine cross striation with a period of about 400 Å in the direction of the fibril axis. However, this feature has not been found sufficiently reproducible or regular to be designated as a periodic spacing in the nature of those found in collagen and in clam muscle fibrils.

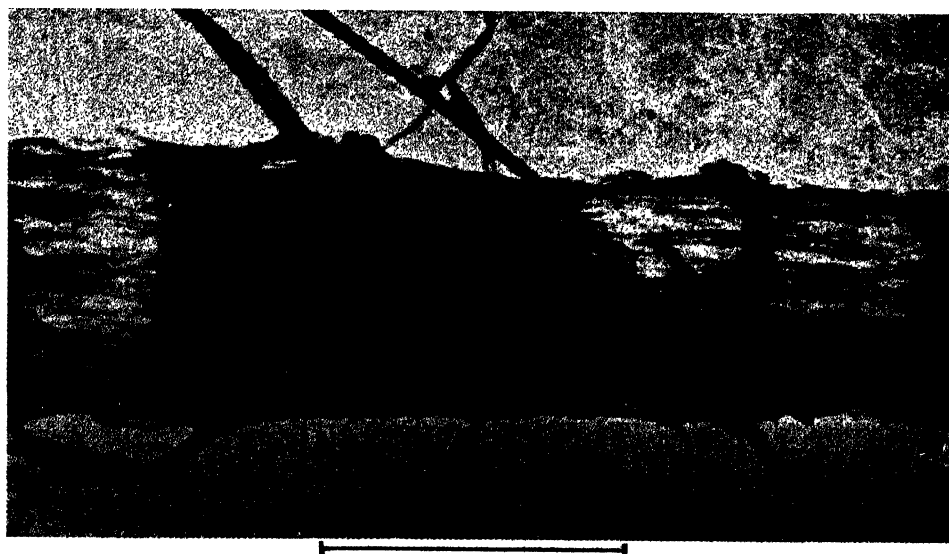
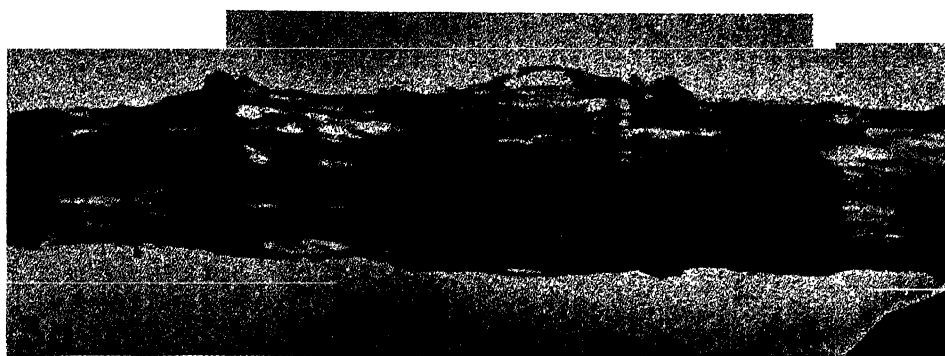
The higher density of the *A* band in electron micrographs of unstained fibrils is in accord with the fact that the refractive index of *A* is higher than that of *I*. Since the myosin filaments pass continuously through both bands, it may be concluded that some other substances occur in much higher concentration in *A* than in *I*. This conclusion is in agreement with the observations of Scott (1932), who found a higher concentration of salts in the *A* band, and of Macallum (1905), who demonstrated a distribution of potassium within the *A* band which is remarkably like the distribution of unstained density as seen in Figure 1. Thus it seems quite evident that the *A* band contains, besides myosin filaments, some substance characterized by a relatively high concentration of salts. This material will be designated as the "*A* substance". Phosphotungstic acid is apparently absorbed in proportion to the density of the *A* substance. It should be noted that phosphotungstic acid forms an insoluble complex with potassium and has been used in the quantitative determination of potassium by Rieben and Van Slyke (1944). This does not mean that the phosphotungstic acid locates potassium specifically for it may react with numerous substances; nevertheless, the observed absorption of this stain in the *A* band is consistent with the conclusion that the *A* substance does contain a relatively high concentration of potassium. That the *A* substance is closely associated with the myosin filaments is indicated by the lack of any observable quantity of interfilamentary material in frayed *A* bands.

The sharp boundary between the *A* and *I* bands is noteworthy since there is no apparent membrane or other structure to confine the *A* substance. Owing to the limited resolution of the light microscope it had been concluded (Schmidt, 1937) that the transition between the *A* and *I* bands is not abrupt but gradual. Likewise there is no evidence in electron micrographs for any envelope or limiting membrane around the myofibril.

One of the most prominent histological features of striated muscle is the *Z* membrane, the nature of which has been the subject of much discussion in the past. In electron micrographs the *Z* membrane appears to be amorphous material of high staining affinity which cements the myosin filaments together in this region. Frequently in frayed myofibrils the filaments separate laterally but adhere to one another in the region of *Z* and also at *M*. Furthermore, there is a tendency for the filaments to break at *Z* and sometimes at *M*. It has been postulated that the *Z* membrane takes the form of an annular ring about the fibril (see Liang, 1936). Although this could be true, there is no unequivocal electron microscope evidence for such a conclusion. The *Z* membrane appears to consist of interfilamentary material present throughout the fibril and not limited to the periphery. It is definitely not collagenous as was suggested by Häggqvist (1931).

FIGURE 3. Myofibrils from frog sartorius, stretched about 30 per cent, stained with phosphotungstic acid; (c) shows collagen fibrils. $\times 40,000$.

PLATE II



Collagen in muscle

In the preparation of specimens no effort was made to separate the collagen, which occurs in skeletal muscle in appreciable quantity. However, there is no difficulty in identifying collagen since it has a regular spacing of about 640 Å and displays a characteristic fine structure after staining with phosphotungstic acid (Schmitt, Hall, and Jakus, 1945). Several stained collagen fibrils are visible in Figure 3c. It has been observed that collagen fibrils from rabbit and frog muscle are quite uniform in width (about 500 Å), but the relation of this protein to the muscle structure is not evident in present electron micrographs because of the extensive fragmentation of the muscle.

Structural alterations in extension and contraction

Although evidence is not yet sufficient to permit a complete description of the structural alterations associated with extension and contraction, some observations have been made and may be described. Extended sarcomeres were obtained by stretching an excised frog sartorius and fixing it at the stretched length. In some instances the muscle was stimulated electrically during fixation to produce isometric contraction. In the absence of the electrical stimulus, the fixative itself provided a weakly stimulating effect. In other experiments, excised muscles were placed in formalin without restraint, thus producing a state of weak isotonic contraction. Strong isotonic contraction was produced by electrical stimulation of excised muscles before and during fixation. Myofibrils from these muscles were prepared for electron microscope observation in the manner described.

The high concentration of *A* substance toward the ends of the *A* band, resulting in the appearance of the *H* disc, occurs consistently in myofibrils from muscles which have been stimulated (electrically or by the fixative) while held in a state of extension (Figs. 1 and 3). Muscles which are free from tension and are stimulated to contract during fixation do not in general show a division of the *A* band. Myofibrils from such muscles are shown in Figure 2.

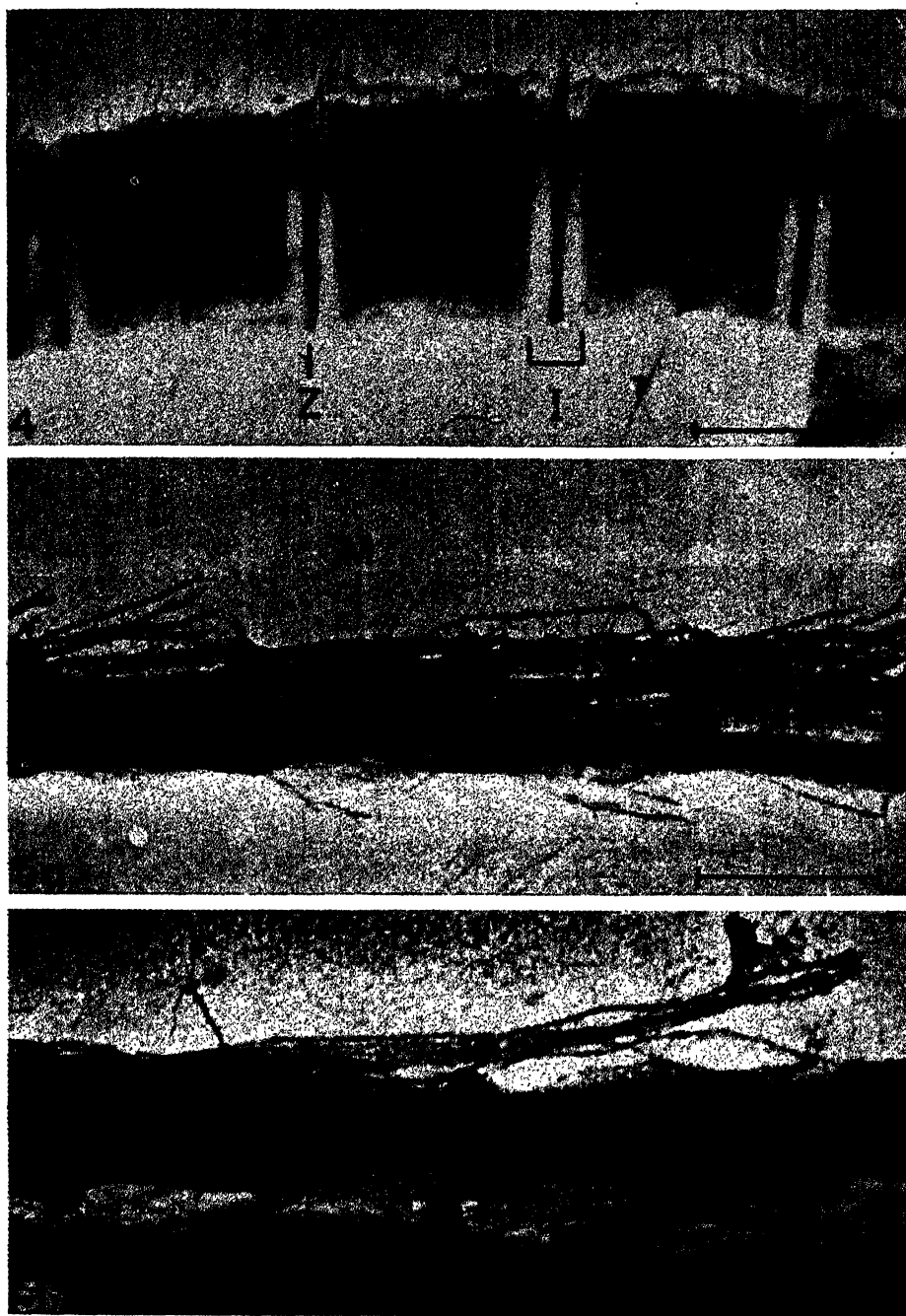
In the myofibrils of muscles which were stretched up to 130 per cent of rest length and fixed at this length, the *A* bands are about the same length as those in muscles which had contracted slightly as a result of fixation. Thus the increase in sarcomere length in stretched and fixed muscles is due mainly to an increase in the length of the *I* band. This observed relative constancy in the length of the *A* band during extension is in agreement with the findings of Buchtal, Knappeis, and Lindhard (1936) who concluded that the *I* band has a relatively low modulus of elasticity.

Electron micrographs of myofibrils from muscles fixed in a state of strong contraction show sarcomere lengths down to about $1\ \mu$ as compared with about $2\ \mu$ in relaxed or weakly contracted muscles. In general the observed sarcomere structure is of two types. The first type, shown in Figure 4, consists of a very narrow *I* band and a quite solid *A* band in which the median *H* disc is absent or very faint. The sarcomere length in Figure 4 is about $1.5\ \mu$, which is about 25 per cent less than the average length in relaxed muscles. The second type of sarcomere structure

FIGURE 4. Myofibril from frog sartorius contracted by electrical stimulation, stained with phosphotungstic acid. $\times 25,000$.

FIGURE 5. Myofibrils from frog sartorius, strongly contracted by electrical stimulation. (a) $\times 25,000$ (b) $\times 40,000$.

PLATE III



found in strongly contracted muscles is shown in Figure 5. Sarcomere lengths in this case are about 1.2μ , which is about 60 per cent of the average relaxed length. The *I* band is no longer visible and *Z* appears somewhat wider and more poorly defined than in less strongly contracted fibrils. The region between *M* and *Z* is of uniform density and presumably contains a uniform concentration of *A* substance. In occasional fibrils the *Z* and *M* are distinguishable only with difficulty. Such fibrils may represent a transition stage between the two types of fibrils described. If this is the case, the second type of fibril might result from the accumulation, around the *Z*, of *A* substance which had migrated away from *M*. This stage apparently corresponds to the striation reversal described by Jordan (1933) and earlier by Rollett (1891), who refers to this densely staining *Z* as the *C'*, or contraction band.

One aspect of particular significance in strongly contracted sarcomeres is the relative straightness of the myosin filaments, as seen in Figure 5. Since the contracted sarcomeres are as little as 50 per cent of the relaxed length, this can only mean that the filaments themselves shorten in contraction. Similarly, in extension the filaments must individually lengthen. Changes in sarcomere length are not to be associated with any gross spiralling or folding of filaments in the order of dimensions visible in the electron microscope. The myosin filaments are the smallest visible contractile units.

ISOLATED MYOSIN FILAMENTS

Myosin may be extracted from muscle in weakly alkaline salt solutions and these extracts have been shown to contain filaments visible in the electron microscope (Ardenne and Weber, 1941). In order to determine whether visible differences exist between different myosins, a quantitative study of filaments from various muscles was undertaken.

Extracts of myosin were prepared by a method essentially similar to that described by Greenstein and Edsall (1940). Muscles were removed from the animal immediately after death, trimmed, cut into small pieces and blended in a Waring Blendor with about ten parts of a cold solution of KCl (0.5 M) and NaHCO₃ (0.03 M). The suspension was stirred mechanically, at about 4° C., for periods of time varying from seven to twenty-four hours and strained through several layers of closely-woven cheese-cloth. The filtrate, showing strong double refraction of flow, was poured into 8 to 10 volumes of cold distilled water, with constant stirring, and the precipitate which formed was allowed to settle overnight in the cold. Further concentration was accomplished by centrifugation, after removal of the supernatant. The precipitated myosin was washed with cold distilled water, and redissolved by adding powdered KCl crystals to a concentration of about 0.45 M. To further purify the myosin the precipitation and solution was repeated once or twice.

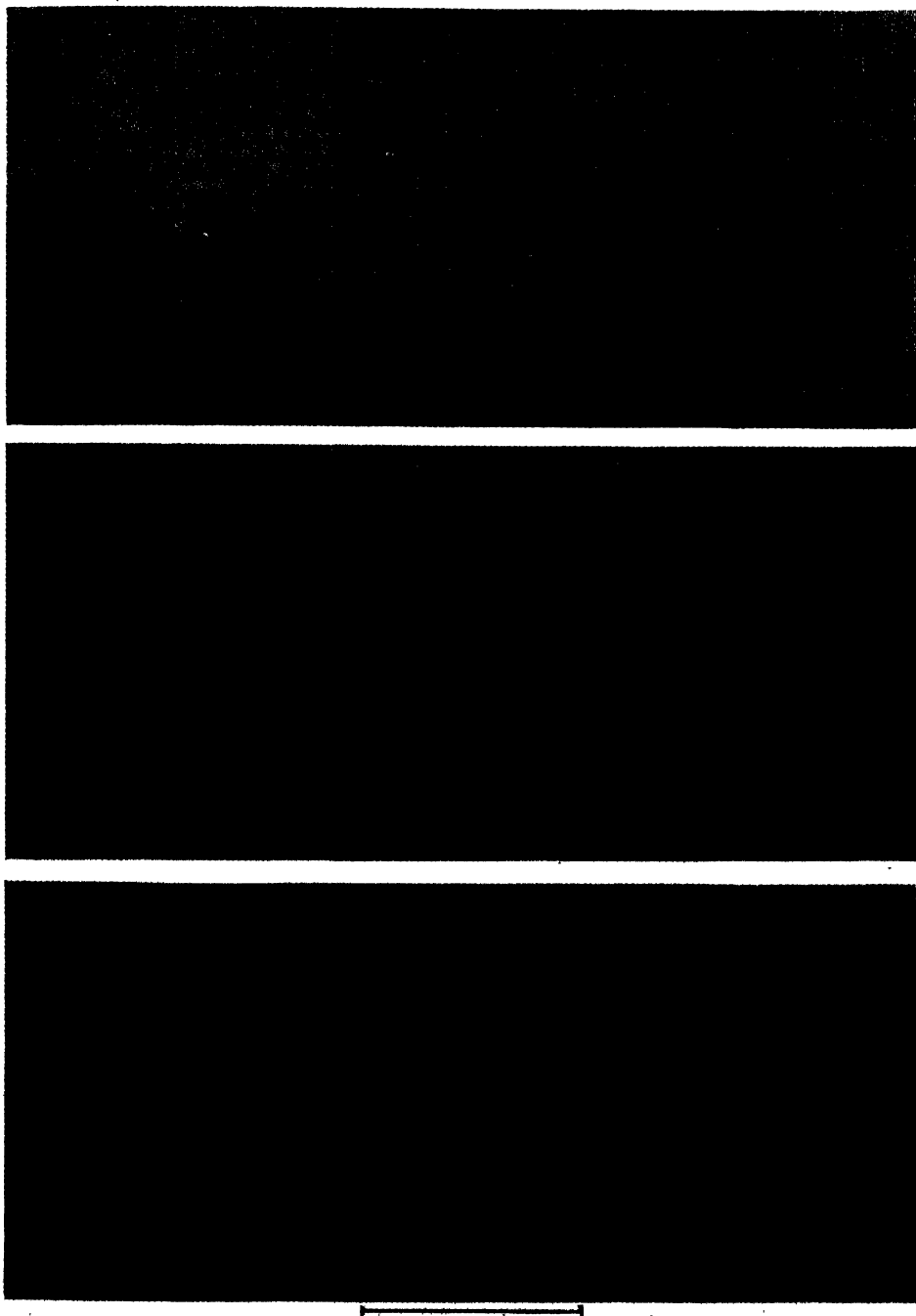
For electron microscope examination, the myosin preparation was diluted to the desired concentration with the KCl-NaHCO₃ solution used in the extraction and centrifuged to remove any undissolved protein. A droplet of the solution was

FIGURE 6. Myosin filaments from rabbit leg muscle. $\times 30,000$.

FIGURE 7. Myosin filaments from lobster abdominal muscle. $\times 30,000$.

FIGURE 8. Myosin filaments from clam (*Mya arenaria*) adductor muscle. $\times 30,000$.

PLATE IV



placed on the supporting film of the specimen grid, the excess removed by blotting, and the film washed with Edsall's solution. The adhering filaments were then fixed and stained, for about one minute, with 0.1 per cent phospho-12-tungstic acid (pH 3 to 5) and washed briefly with water to remove the uncombined phosphotungstic acid. Unstained filaments are poorly defined because of low contrast and, in the absence of the stabilizing effect of the heavy metal ion, appear to be adversely affected by the drying process.

Myosin extracts were made from the muscles of rabbit leg, frog leg, lobster abdomen, scallop adductor (striated part) and clam (*Mya*) adductor (classified as smooth muscle). Electron micrographs of such myosin preparations show slender filaments similar to those observed in intact myofibrils and resembling those shown by Ardenne and Weber (1941). The filaments have varying lengths and widths and display no tendency to branch or split longitudinally. They are rough in appearance and show fluctuations in density along their length. However, there is no evidence of any regular structural variation which could be interpreted as a significant periodicity. There are no observable changes in density which can be correlated with the *A* and *I* bands, which indicates that the *I* substance has probably been washed out during extraction. This general appearance is very much the same for myosin filaments from the five different animal forms. Typical electron micrographs of myosin filaments from rabbit, lobster, and clam are shown in Figures 6, 7, and 8 respectively.

A statistical study of lengths and widths of myosin filaments from the five selected forms was made. Measurements were taken directly from enlarged prints and in any given print all filaments were measured which could be discerned as complete and individual. Widths can be measured only approximately because of the smallness of this dimension and also because of the roughness of contour. The uncertainty of measurement, however, is significantly smaller than the spread in widths. Results are shown in Figure 9. Each interval in the plot of widths contains the same number of measurable increments and each set of measurements represents about 300 filaments.

TABLE I
Dimensions of myosin filaments

Muscle source	Average width	Average length
Rabbit leg	120 Å	4100 Å
Frog leg	140 Å	4100 Å
Lobster abdomen	140 Å	6800 Å
Scallop adductor, striated	130 Å	5000 Å
Clam (<i>Mya</i>) adductor, smooth	150 Å	3100 Å

Nearly all measured widths fall into a narrow range between 50 and 300 Å. There may be some filaments having widths below 50 Å which were not observed because of the resolution and contrast limitations of the electron microscope. However, the quality of the background in the electron micrographs and the fact that all distributions fall off toward small widths indicate that no great quantity of the myosin occurs in this range in electron microscope specimens as prepared.

Between one animal form and another there is very little variation in magnitudes or distribution of widths. There is a slight difference in the averages as shown in Table I, but it is so small as to be scarcely significant.

Filament widths were also measured from electron micrographs of intact myofibrils from frog muscles. The distribution plot resembles very closely the corre-

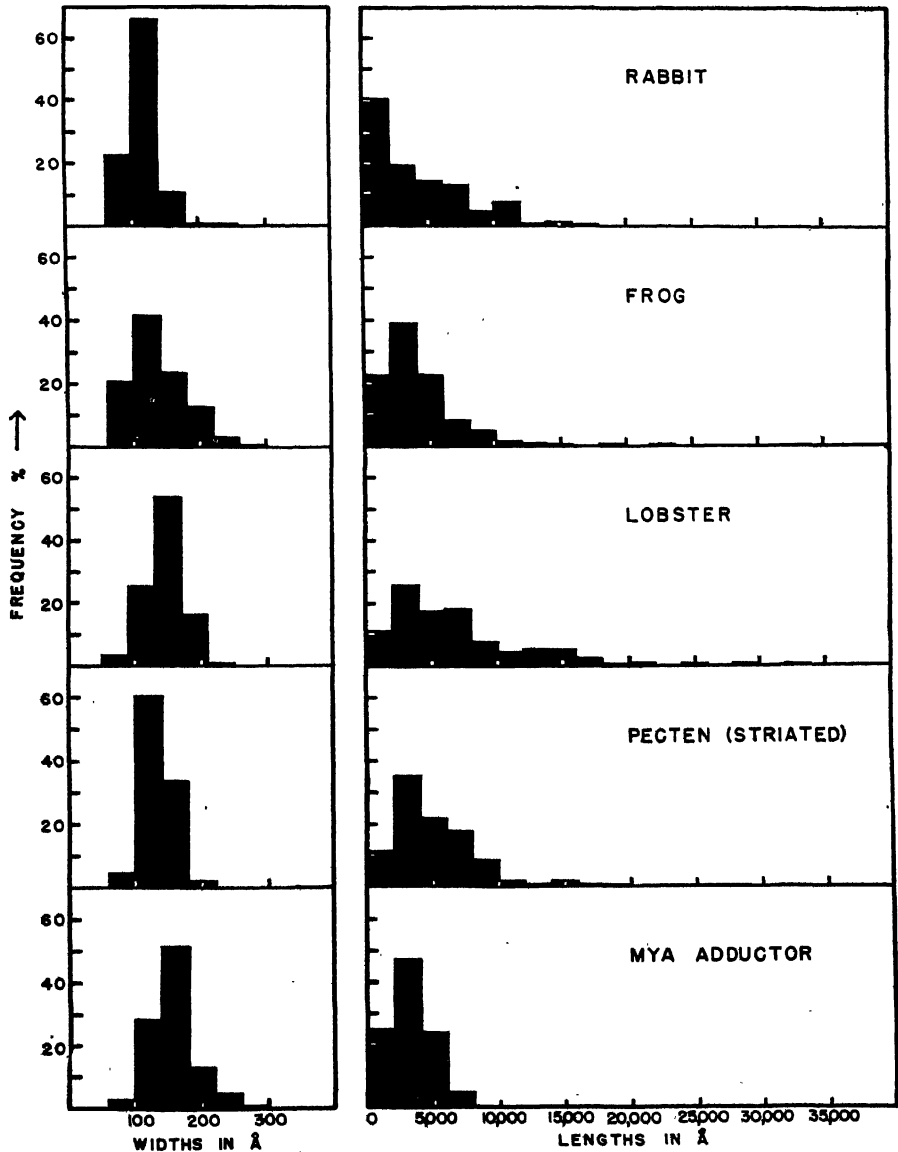


FIGURE 9. Distribution of lengths and widths of myosin filaments from muscles of various animal forms.

sponding plot for frog shown in Figure 9, thus indicating that there is no great difference in the width of filaments in electron micrographs of intact fibrils and myosin extracts.

In comparison to the fair constancy of widths there is a wide distribution of lengths. Most of the measured lengths are below 15,000 Å. Filaments from lobster muscle are in general significantly longer than the others, while filaments from *Mya* adductor, which is the only smooth muscle studied, are significantly shorter. As a result of the wide range of lengths there is only rough significance in the number-averages given in Table I. The distribution plots are reasonably reproducible under similar conditions.

It is evident from the statistical results that myosin extracts of this type do not constitute a monodisperse system and there is no justification for referring to the filaments as molecules. Widths of filaments apparently do not alter appreciably during extraction. The lengths, however, bear no relation to any observable dimensions in the intact myofibrils. Since most of the lengths represented in the plots of Figure 9 are considerably shorter than are the corresponding sarcomeres, it is apparent that the myosin filaments have been broken at random into shorter segments during the extraction procedure.

Since the myosin filaments seen with the electron microscope are to be identified with the asymmetrical particles responsible for the streaming double refraction of myosin solutions, it is pertinent to compare the results of the methods where possible. Mehl (1938) reported the length of rabbit myosin as 8,500 Å while Edsall (1942) gives a figure of 12,600 Å. The second figure is near the upper limit of the distribution plot for rabbit in Figure 9 while the first falls within the range of lengths representing the bulk of the protein. Although no figures have been reported on the other muscles used here, Edsall and Mehl (1940) have described lobster myosin as being more viscous than rabbit myosin at equal concentration and strongly birefringent, which is in qualitative agreement with the electron microscope observation that lobster preparations contain significantly longer fibrils than do those from rabbit.

Ardenne and Weber (1941) do not give the source of the myosin extract from which they made electron micrographs. They state that the filament widths are from 50 to 100 Å although no measurements are tabulated and the micrographs used as illustration contain filaments at least 200 Å in width. The myosin filaments within the limited field of the micrographs which these authors have shown are apparently similar to those described here.

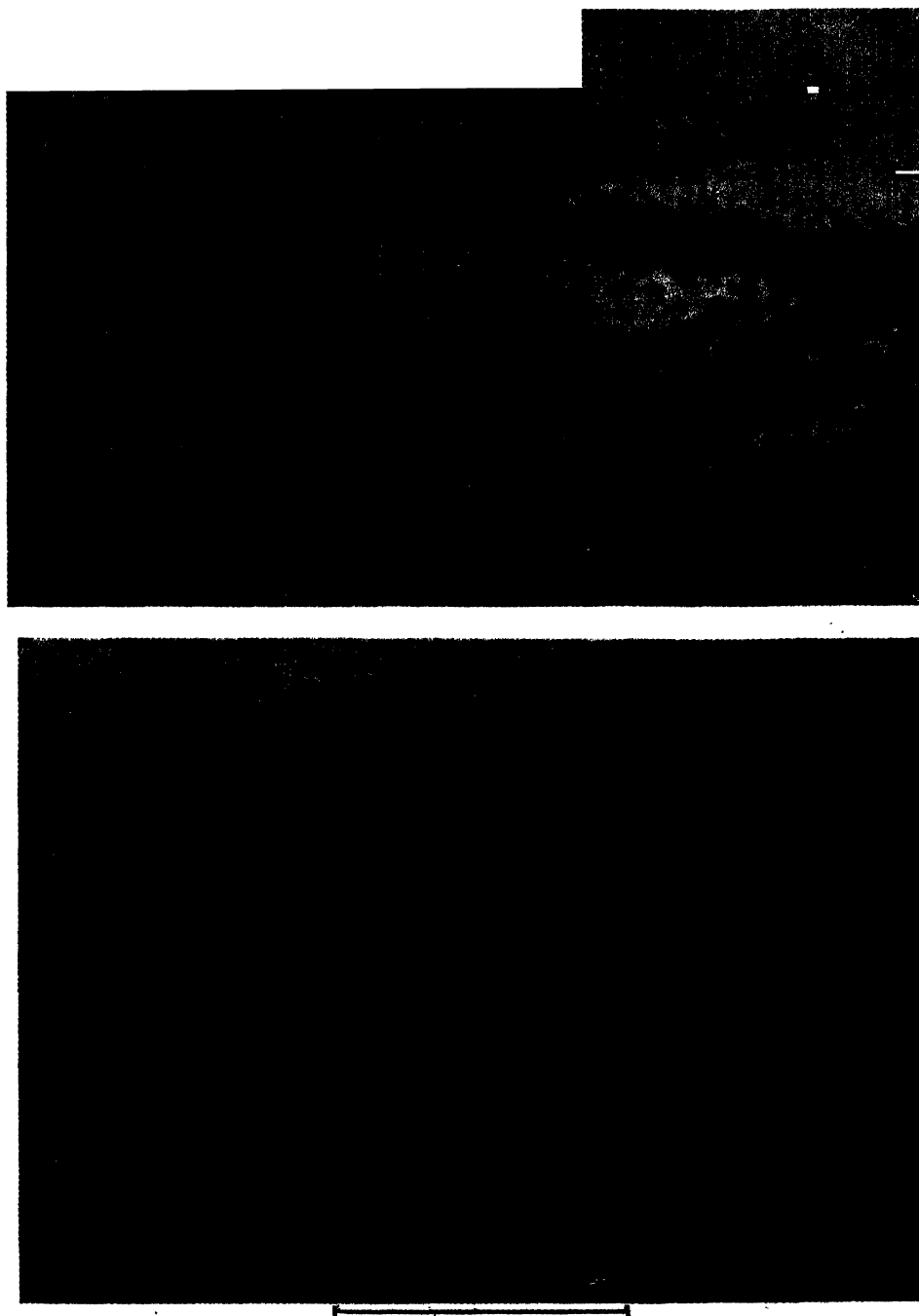
Paramyosin

In a recent publication Hall, Jakus, and Schmitt (1945) described the structure of a fibrous muscle globulin which is present in appreciable amount in molluscan smooth muscles, and correlated the observed structure with the x-ray patterns obtained by Bear (1944) from the same material. Since this protein can be identified by electron microscope observation and x-ray diffraction it merits a distinguishing name and is therefore designated as *paramyosin*. Although paramyosin

FIGURE 10. Regular structures from clam adductor muscle after dispersion in Edsall's solution. $\times 40,000$.

FIGURE 11. Fragments from dispersed clam muscle. Darkly staining particles are adhering to fine filaments, presumably myosin. $\times 40,000$.

PLATE V



resembles myosin in some respects, there are definite differences between the two proteins. Paramyosin contains a characteristic axis spacing of about 145 Å. Paramyosin has not been detected in striated muscle while myosin is apparently common to all muscles. Furthermore, the typical needle-shaped fibrils of paramyosin disintegrate in Edsall's solution (0.5 M KCl, 0.03 M NaHCO₃) while the myosin filaments remain essentially the same as in fixed intact muscle except as they may be broken into shorter lengths during extraction.

Clam adductor muscles

The adductor muscle of the clam differs from the other muscles investigated in that it is classified histologically as "smooth" muscle and contains a large quantity of the fibrous protein, paramyosin. Electron microscope observation has failed to reveal any large periodicity resembling the characteristically banded sarcomere of striated muscle. However, if clam muscle is dispersed in Edsall's solution and centrifuged, there are thrown down fibrous aggregates to which large particles adhere. A typical micrograph of this type of material is shown in Figure 10. The structure consists essentially of bundles of fine filaments (presumably myosin) and darkly staining nodules producing a cross striation with a period of about 1,100 Å. In Figure 11 the bundles have been dispersed, revealing the individual dark nodules which adhere to the fine filaments at more or less regular intervals. The attachment of the dense component at regular intervals along myosin bundles (Fig. 10) is suggestive of a rudimentary structure analogous to the *Z* membrane and sarcomere of striated muscle. As yet, the significance of this structure is not known.

DISCUSSION

With respect to the existence and disposition of the cross striations, the electron microscope observations on fixed muscle, stained and unstained, are in close agreement with the results obtained with the light microscope (see Jordan, 1933). Confirmatory evidence is presented concerning the *N* bands, *H* discs and the nature of "striation reversal" in strong contraction. Additional information has been added by virtue of the high resolution of the electron microscope. Thus it is possible to say definitely that the *Z* band is not collagenous as suggested by Häggqvist (1931) and to observe directly the myosin filaments and their relation to previously determined structures.

Of fundamental importance is the observation that the myosin filaments extend continuously through the fibril in relatively parallel straight lines. There is no marked disorientation of the filaments in either *A* or *I* bands of fibrils in any state of contraction. Although the optical anisotropy of the *A* band led to the postulate that it contains asymmetric myosin particles, there has always been some doubt as to whether the relatively isotropic *I* band consists of myosin in unoriented state or of some other protein (Weber, 1934). It is now clear that the *I* band consists mainly of well oriented myosin filaments and it is not possible to account for the low birefringence on the basis of gross disorientation. Possibly the difference in anisotropy between the *A* and *I* bands is to be attributed to differences in orientation within the filaments. The only obvious alternative explanation is that the low birefringence in the *I* band is due to partial compensation of the birefringence of the myosin filaments by other components.

No evidence has been found in this investigation for the existence, either in myofibrils or in extracted myosin, of "rodlets" of the specific dimensions postulated by Weber (1934) from polarized light and diffusion experiments. Theoretical difficulties underlying Weber's calculations have been pointed out by Frey-Wyssling (1940) and Schmitt (1944).

Electron micrographs of isolated myosin filaments show that in width and general appearance the filaments are similar to those seen in fixed intact myofibrils. Although myosin filaments from whatever source are essentially the same, the statistical study indicates that they occur in various lengths and are not to be designated as discrete "myosin molecules". However, the length of rabbit myosin reported by Mehl (1938) from streaming double refraction studies is reasonably close to the weight-average length calculated from the distribution curve in Figure 9. No reliable measurement of width is available for comparison with the electron microscope results.

Since myosin filaments in intact fibrils are continuous, the wide range of lengths found in myosin suspensions is noteworthy. It appears that the filaments, while little changed in width, are broken more or less at random during the extraction procedure. The longest filaments approach the sarcomere length and there may be significance in the fact that lobster muscle yields longer filaments than does frog muscle and also has the longer sarcomere. The absence of filaments longer than their corresponding sarcomere is consistent with the observed tendency of the filaments to break at Z.

Ziff and Moore (1944), following an extraction procedure similar to that used here, state that their myosin solutions contain a homogeneous substance which forms sharp boundaries in electrophoresis and sedimentation. If homogeneity is meant to denote constancy in particle length, this conclusion is in disagreement with the electron microscope results. In considering the apparent inconsistency it should be noted that, in the electrophoresis and sedimentation of rod-shaped particles of nearly constant diameter, a sharp boundary does not necessarily indicate a constant length. Schramm and Weber (1942) reported that a small fraction of the myosin in extracts has a much higher sedimentation constant than that of the predominant component. No evidence for such a distinct heavy component has been found in the present study unless it represents aggregates of myosin filaments.

The "molecular weight" of myosin has been estimated by Weber and Stöver (1933) to be $0.6 - 1.2 \times 10^6$ and by Ziff and Moore (1944) to be 3.9×10^6 . If a particle weight is calculated from the average dimensions of the filaments in rabbit myosin from Table I, using a density of 1.3 and assuming a circular cross section, the result is 36×10^6 . The results differ by at least a whole order of magnitude. In view of the fair agreement as to filament lengths from streaming birefringence and electron microscope observations, the discrepancy in particle weight is difficult to understand. It may be that the cross section of filaments dried on the supporting film is not circular as assumed in the electron microscope calculation, but it is doubtful whether this assumption could introduce an error large enough to account for the discrepancy. In any event, the significance of all such calculations is questionable inasmuch as the extracts contain particles of widely differing dimensions, in no sense to be considered as molecular entities.

Bear has reported small-angle x-ray diffractions from various muscles. One set of diffractions (Bear, 1944) has been correlated with the structure of para-

myosin fibrils, as determined from electron micrographs (Hall, Jakus, and Schmitt, 1945). This set of diffractions has not been obtained from any striated muscle.

In addition Bear (1945) has reported a second set of small-angle diffractions obtained from a variety of muscles, both smooth and striated. The wide occurrence of this pattern is strongly suggestive that the diffractions originate in the myosin component but it has not been possible to identify in the electron microscope the structure responsible for the diffractions. Furthermore, since the meridional diffractions are orders of 27 \AA , it is doubtful whether the structure can be observed directly. Another feature of the diffractions is the occurrence of a periodicity estimated by Bear to be between 350 and 420 \AA . Although this dimension is quite large enough for electron microscope resolution, it appears that the pattern is somewhat like the paramyosin pattern in that the large periodicity cannot be discerned unless the axis spacing (27 \AA) is well resolved. The fine banded appearance frequently observed in myofibrils is of about the same order of magnitude as the large periodicity, but this may be fortuitous. The x-ray data also indicate a lateral periodicity of about 115 \AA which should be large enough for electron microscope observation. Although the *average* width of myosin filaments is quite close to this figure, the significance, if any, of this coincidence remains to be determined.

Since the myosin filaments are observed to pursue a straight course through the *A* and *I* bands in fibrils from contracted as well as relaxed muscles, it may be concluded that contractility is a property of the individual filaments in their normal environment in muscle. It seems probable that alterations in length and tension depend on changes within the filaments in response to changes in the chemical environment. In seeking a description of the contractile mechanism, due consideration must be given to the role played by the *A* substance and the adenosine triphosphate. The electron microscope technique provides a promising method for studying these structures and processes. It may be expected that further correlation of the x-ray, electron microscope, polarization optical and chemical evidence will contribute greatly to an understanding of the nature of contractility.

SUMMARY

1. Electron micrographs were made of myofibrils isolated from frog and other skeletal muscles fixed in formalin. The structure with respect to the location and disposition of the principal cross striations is in good agreement with that previously determined from histological studies.

2. The myofibrils are composed of bundles of myosin filaments ranging in width from about 50 to 250 \AA and extending continuously and in relatively straight lines through the isotropic and anisotropic bands in both the extended and contracted states. The anisotropic bands also contain material of high electron scattering power and affinity for phosphotungstic acid. The distribution of this "*A* substance" changes with contraction in characteristic fashion. The evidence indicates that the myosin filaments are the contractile units.

3. While the myosin filaments have an indefinite length in the intact fibril, they are fragmented extensively during extraction in weakly alkaline salt solutions (method of Greenstein and Edsall). Filaments from such extracts have fairly uniform widths (50 to 250 \AA) but highly variable lengths, in general below 15,000 \AA . Filaments from rabbit, frog, lobster, scallop and clam muscles are similar in appear-

ance; widths are fairly uniform but lengths vary significantly from one form to another.

4. The relation of these findings to physical chemical data previously obtained by others on similar myosin extracts is discussed.

5. In the one smooth muscle examined (clam adductor) no striations comparable to those of skeletal muscle fibrils were found. However, a regular structure with a period of about 1,100 Å was observed.

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EFFECT OF WATER CURRENTS UPON THE ATTACHMENT AND GROWTH OF BARNACLES¹

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INTRODUCTION

There exists a considerable body of published work dealing with peculiarities in the distribution of sessile marine invertebrates in general, and, although experimental evidence is incomplete some speculation has been offered as to the manner in which their distribution is limited by various factors of the environment which influence their attachment and growth. The object of the present studies was to investigate the action of the water current factor by experimental methods, with particular reference to the species of barnacles which are most abundant in the Miami area.

Much of the earlier work on sessile marine animals has been reviewed by Mc Dougall (1943), and need not be considered here in detail. A bibliography of investigations dealing with sessile organisms from the point of view of ship fouling is given by Neu (1933), and an excellent general account of marine fouling by Visscher (1928) who does not, however, include more than a brief reference to the effect of water currents.

The effects of environmental factors have been deduced from the results of ecological surveys by a number of investigators. Stephenson and his co-workers, in a series of publications entitled *The South African Zone and Its Relation to Water Currents* (see bibliography), Fischer-Piette (1928, 1928a), Pierron and Huang (1926), and Prennant and Teissier (1924) have described the populations of rocky shores and the distribution of sessile organisms in general, but with scanty reference only to the relation between barnacle attachment and the velocity of water currents. Stephenson and Bright (1938) and Stephenson and du Toit (1937), however, note the absence of certain species of barnacles from surfaces exposed to heavy wave action.

The possibility of water movement influencing barnacle attachment is suggested by Fischer-Piette (1932) in his reports on surveys of the shores of the English Channel. Shelford and Towler (1925), Towler (1930), and Newcombe (1935) have paid particular attention to barnacles in associations of *Balanus-Littorina* and *Balanus-Mytilus* and draw attention to the more luxuriant growth of barnacles exposed to strong water currents, particularly in shallow water. The role of water currents is also mentioned briefly in accounts of ecological surveys by Rice (1930), Moore (1935a), and Moore and Kitching (1939), who stress the part played by

¹ These experiments were conducted while the author was engaged by the Woods Hole Oceanographic Institution in investigations under contract with the Bureau of Ships, Navy Department, which has given permission to publish the results. The opinions contained herein are those of the author and do not necessarily reflect the official opinion of the Navy Department or of the Naval service at large.

² Contribution number 301 from the Woods Hole Oceanographic Institution.

them in the provision of an adequate food supply, as well as the adverse effects of wave action.

Experimental investigation has yielded more direct information. Moore (1933) studied the effect of currents upon the attachment of cyprid larvae with particular regard to orientation, but gives no estimate of current velocities which limit attachment. The related phenomena of wave action are considered by Moore in a separate paper (1939).

Evidence as to the effect of water currents is also afforded by the study of ships and other objects moving through the water. The economic importance of ship-bottom fouling has stimulated this kind of observation. Heuschel (1934) and Neu (1932) state that vessels collect barnacles most readily when in harbor and when subjected to relatively less water movement. Visscher (1930) further observes that barnacles are usually killed during ocean passages of about 500 miles. Hiraga (1934) showed experimentally that, in the laboratory, 10 to 17 day old barnacles were lost during a five day exposure of test planks to water currents, whereas twenty-four day old barnacles remained alive and intact. In a later paper of Visscher (1938) he concludes that barnacles are not distributed by ships. This is contrary to the views of Fischer-Piette (1929, 1935) who expresses the opinion that *Balanus amphitrite* found by him on the coast of France, was brought from the tropics on the hulls on ships. Later observations by Phelps (1942) on test panels support the conclusion that excessive water currents inhibit the attachment and growth of barnacles.

The extensive observations of McDougall on the pile fauna at Beaufort include the results of exposing various types of experimental collecting apparatus. His experiments on the effect of water currents indicate that fewer barnacles settle and that growth is slower in more rapid currents. His results did not, however, give an accurate indication of limiting or optimum velocities to which his collectors were exposed.

The experiments which form the subject of the present account are divided into a study of the effects of water currents upon the attachment of cyprid larvae, and into a consideration of the effects of water currents upon the organisms following attachment. Under natural conditions of fluctuating tidal currents and wind drifts it is conceivable that a cyprid might attach during a period of minimum water flow in a particular region and that, once attached, the maximum flow would be insufficient to detach it or to inhibit its growth. The aim of the investigation was to establish the limiting velocities of current for initial and continued attachment and growth.

The advice and suggestions of Dr. Alfred C. Redfield of the Woods Hole Oceanographic Institution are gratefully acknowledged. The writer is also indebted to Mr. Frank L. LaQue of the Development and Research Division, International Nickel Company, Inc. and to Dr. William F. Clapp for permission to include in this paper data from their observations at Kure Beach, N. C. Acknowledgments are also due Mr. D. S. Reynolds, Mr. James Gregg, Mr. Alexander Frue, and Mr. Charles Weiss for their assistance at various times.

METHODS

In order to study the effects of water currents two types of apparatus were designed which would permit the movement of sea-water relative to the experimental

surface at predetermined and variable velocities. The first consisted of a rotating disc, immersed in the sea. The second type of apparatus consisted of a series of glass tubes of varying cross-sectional diameter. The rotating disc was employed both in the study of initial attachment of barnacles and in the study of growth subsequent to attachment. The glass tubes of graded diameter were employed in the observations on initial attachment only, as a check against results obtained with the rotating disc.

WATER CURRENTS AND ATTACHMENT ON THE ROTATING DISC

The rotating disc apparatus as used in the experiments here described is essentially similar to a machine described by LaQue (1943) for the purpose of studying the effect of sea-water currents upon corrosion of metal samples, with certain changes in design appropriate to the particular use to which it was put. The modified machine consists of a vertical shaft rotated by means of an electric motor and a system of belts and pulleys. The disc is attached to the shaft by means of a flange and small brass screws and is maintained in a position several inches below low water spring tide. At velocities of rotation used in the experiment no cavitation occurred at this depth.

By changing the arrangement of pulleys the speed of rotation may be varied up to approximately 1750 r.p.m. The rate of movement in knots of any portion of the disc relative to an imaginary stationary body of water is readily calculated as approximately $R \times D/370$, where R is the number of revolutions per minute and D the diameter in inches. This velocity is nominal, however, and does not accurately represent the rate of relative movement between the disc and the water close to its surface. Frictional drag and centrifugal forces produce vortex movements which cause water to flow in a close spiral roughly parallel to the plane of the disc. As a result, the actual flow of water at any part of the disc's surface is less than the nominal rate calculated from the velocity of rotation. Size of the discs used varied up to 28 inches in diameter.

Experimental procedure consisted of bolting a new rotating disc on the shaft and setting the machinery in continuous operation. The disc was removed and examined at suitable intervals. At the same time a similar stationary disc was immersed at a point nearby the rotating disc, at the same depth and resting similarly in a horizontal plane. Observations on the second disc acted as a control for such factors as abundance of organisms, nature of the surface of the disc, etc. The apparatus was used at the edge of a covered slip at the Miami Beach Boat Corporation, where fouling is usually severe. The predominant organism at most times of the year is *Balanus amphitrite niveus* Darwin and this was accordingly selected for observation.

Results

The results of the first rotating disc experiment indicated that the approximate minimum current velocity required to prevent barnacle attachment is 1.1 knots. The disc used was 13 inches in diameter and was rotated at 540 r.p.m. The disc, together with its stationary control, are shown in Figure 1 as they appeared at the end of this experiment. Five barnacles attached to the center of the rotated disc during 23 days but did not grow beyond 2 mm. in greatest width. The limiting velocity was calculated from the $\frac{3}{4}$ inch diameter of the circle to which the barnacle attachments were restricted (Table I).

TABLE I

Effect of water currents upon attachment of Balanus amphitrite as observed on the lower surface of a horizontal rotating disc

Disc	Date of start	Duration of rotation (Days)	Speed of rotation (r.p.m.)	Maximum diameter at which attachment occurred (Inches)	Maximum nominal velocity at which attachment occurred (Knots)
1 B	8/20/43	23	540	$\frac{3}{4}$	1.1
4 A	11/4/43	16	192	2	1.0
6 A	12/7/43	14	134	$2\frac{1}{2}$	0.9
7 A	1/5/44	19	60	8	1.3
Average	—	—	—	—	1.1

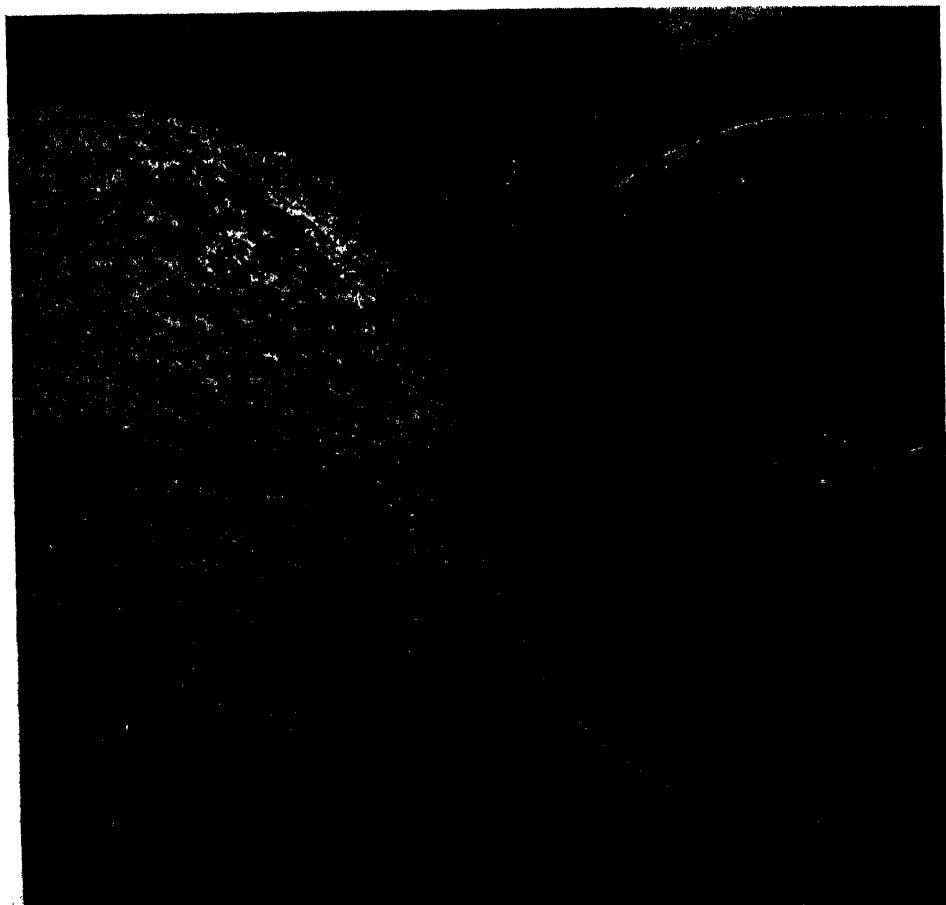


FIGURE 1. Surface of disc rotated at 540 r.p.m. for a period of 23 days, together with stationary control. Diameter of disc, 13 inches.

In order to establish the limiting velocity with greater accuracy the experiment was repeated on three other occasions at slower speeds of rotation, with the results shown in Table I and Figure 2. The nominal velocity limiting barnacle attachment as shown by these experiments varied from 0.9 knots to 1.3 knots with an average of 1.1 knots. In addition to the adverse effect upon barnacle attachment it was noticed that slime film development was greatly reduced on the rotated disc.

ATTACHMENT IN GLASS TUBES

Owing to the difficulty of accurately measuring the rate of water flow along the surface of the rotating disc, an independent method of investigating the relation between water currents and fouling incidence was used. In this case sea water was passed through sections of glass tubing of varying diameter (Fig. 3). Comparison of fouling incidence in each sector was made with the varying linear velocity of the water, calculated on the assumption that linear rate of flow through tubes varies inversely as the square of the diameter of cross-section. In order to estimate errors which might be introduced by virtue of turbulent flow, carmine was introduced into the water during a preliminary test run. It was observed that flow in the main portion of each section was smooth. Areas near to the joints, where turbulence was noted, were not included in the experimental observations of fouling incidence.

The tubes were approximately eight inches long and of 5 cm., 3.7 cm., 2.8 cm., 2.2 cm., and 1.4 cm. internal diameter respectively and were joined by means of fitted rubber stoppers. Attachment of barnacle larvae was encouraged by excluding direct sunlight. The water flow was provided by means of a centrifugal pump, with the experimental tubes arranged on the inlet side in order to avoid possible mechanical damage to the larvae.

While the apparatus was in operation the rate of flow was checked daily by volumetric measurement at the outlet. At the conclusion of each test run the tubes were carefully examined for signs of fouling.

Results

Observations on the glass tubes showed that limiting velocities for attachment of *Balanus amphitrite* were between 0.5 and 1.0 knot. During the first experiment with this apparatus, sea water was run through it at a rate between 13 and 16 liters per minute, on a catwalk extending over the water at the University of Miami Marine Laboratory. Fluctuations in the height of tide and consequently the suction head of the seawater pump gave rise to this fluctuation in current velocity. Barnacles attached in the two larger tubes within ten days after beginning the experiment. From the calculated linear velocity of the narrower of these tubes it appeared that barnacles are not prevented from attachment by currents varying between 0.5 and 0.6 knot (Table II). In the next smaller tube where no barnacles attached the rate of flow varied from 0.8 to 1.0 knot. The limiting velocity therefore lies between 0.5 knot and 1.0 knot. The appearance of the tubes at the end of a similar experiment is illustrated in Figures 3 and 4. The upper figure of the limits is lower than the critical velocity as measured on the rotating disc. However, in view of the difference in hydrodynamical conditions involved in the two methods, which was discussed previously, the results appear to be reasonably consistent.



FIGURE 2. Portion of disc rotated at 192 r.p.m. for a period of 16 days, showing barnacles attached at center. Diameter of disc, 24 inches.

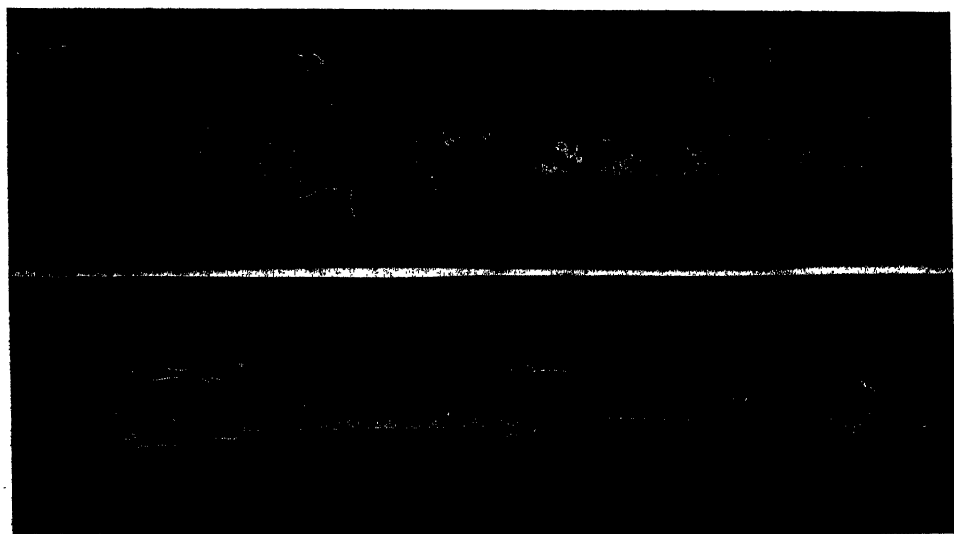


FIGURE 3. Series of five tubes of increasing cross-sectional diameter. Shows development of barnacles in wider tubes only, following prolonged passage of sea-water.

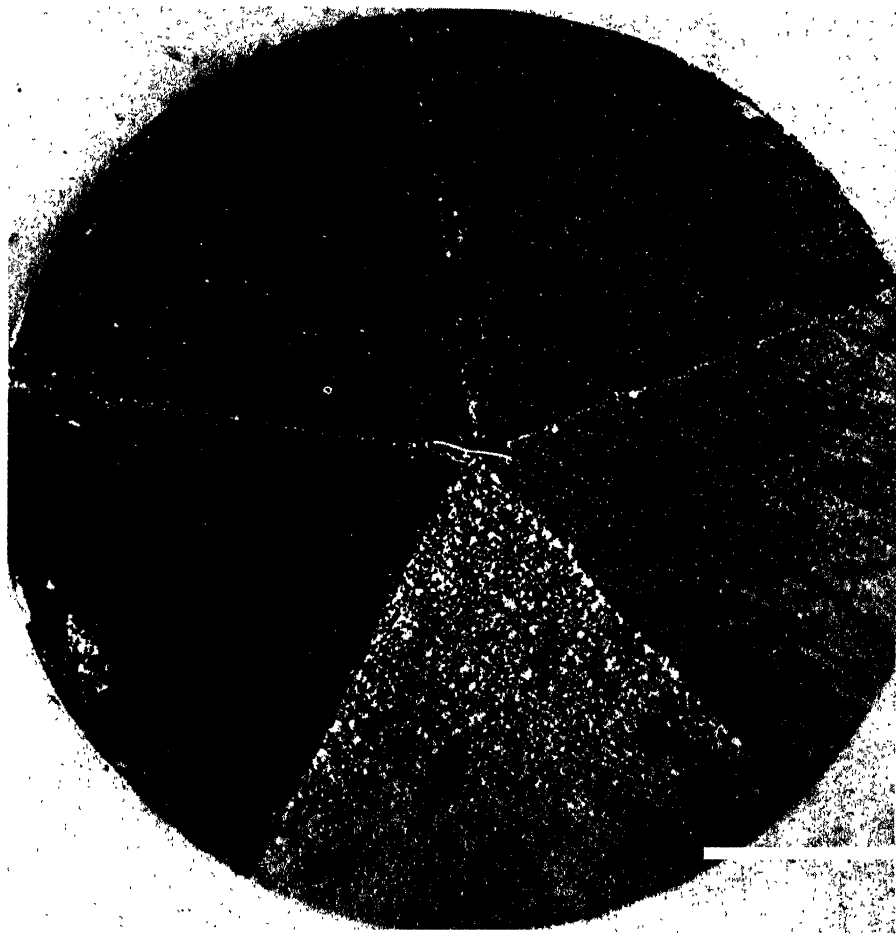


FIGURE 4. Surface of disc following 16 days of stationary immersion beginning January 8, 1944. Individual sectors of the disc were exposed for periods of 6 hrs., 1 day, 2 days, 5 days and 16 days. Diameter of disc, 28 inches.

At a later date the glass tubes were exposed at Kure Beach, N. C., by Mr. F. L. LaQue and Dr. W. Clapp, under conditions allowing a more constant flow of sea water. By reducing the flow of water in successive experiments the critical velocity for attachment was confined more closely between 1.1 and 1.3 knots for *Balanus improvisus* and below 0.8 knot for *B. eburneus*. These results are included in Table II with the permission of LaQue and Clapp.

Confirmatory results have been reported by Turner (1945) from an experiment recently conducted at Kure Beach in which a system of steel pipes of graded diameter was exposed to sea water circulation for 98 days. Fouling with mussels, barnacles, and tube worms occurred where the current velocity was one knot or less but not where it was 1.8 knots or greater.

TABLE II

Attachment of Balanus species upon vertical glass tubes of graded diameter during the passage of sea-water

Period and place	Rate of flow. Liters per minute	Equivalent velocity in glass tubes (Knots)				
		1.4 cm. diameter	2.2 cm. diameter	2.8 cm. diameter	3.7 cm. diameter	5.0 cm. diameter
11/17/43 to 11/27/43 Miami Beach	13-16	3.0-3.7	1.2-1.6	0.8-1.0	0.5-0.6	0.2-0.3
		—	—	—	<i>B. amphitrite</i>	<i>B. amphitrite</i>
5/20/44 to 7/12/44 Kure Beach*	21	5.3	2.3	1.4	0.8	0.4
		—	—	—	<i>Balanus</i> sp.	<i>Balanus</i> sp.
7/12/44 to 9/18/44 Kure Beach*	12.7	3.2	1.3	0.8	0.5	0.2
		—	—	<i>Balanus</i> sp.	<i>Balanus</i> sp.	<i>Balanus</i> sp.
9/18/44 to 11/25/44 Kure Beach*	10.8	2.7	1.1	0.7	0.4	0.2
		—	<i>B. impro- visus</i>	<i>B. impro- visus</i>	<i>B. eburneus</i> <i>B. improvisus</i>	<i>B. eburneus</i> <i>B. improvisus</i>

* Observations by W. F. Clapp and F. L. LaQue.

GROWTH ON THE ROTATING DISC

For the purpose of studying the effect of water currents upon barnacles already attached the rotating disc procedure was modified. Both rotating and stationary discs were allowed a preliminary period of stationary immersion during which barnacles became attached. Equal sectors of the disc were temporarily protected by means of cloth attached with thumb tacks. The cloth was removed from each sector after increasing intervals of time with the result that at the end of the preliminary period of immersion the maximum age of the organisms attached to the different parts of the disc varied from 16 days to 6 hours. The barnacles on each sector were then counted and the maximum diameter of the largest barnacle in each sector was measured.

Following the period of stationary immersion the control disc was allowed to remain in a stationary position and the other was placed on the rotating shaft for a further period. Both discs were examined and the number of barnacles per square inch and their greatest diameter were recorded separately for each sector and for areas at successive distances from the center of the disc.

Results

The first experiment of this series provided an initial 16 days' period of stationary immersion during which both experimental and control discs became seeded

with barnacles. At the end of this period barnacles had been attached on the five sectors for maximum periods of 6 hours, 1 day, 2 days, 5 days, and 16 days respectively (Fig. 4). Immediately following the period of stationary growth, the experimental disc was rotated at an angular velocity of 60 r.p.m. for 19 consecutive days, while the control disc was allowed to remain in a stationary position for the same period. The appearance of both discs at the end of the experiment is shown in Figures 5 and 6.

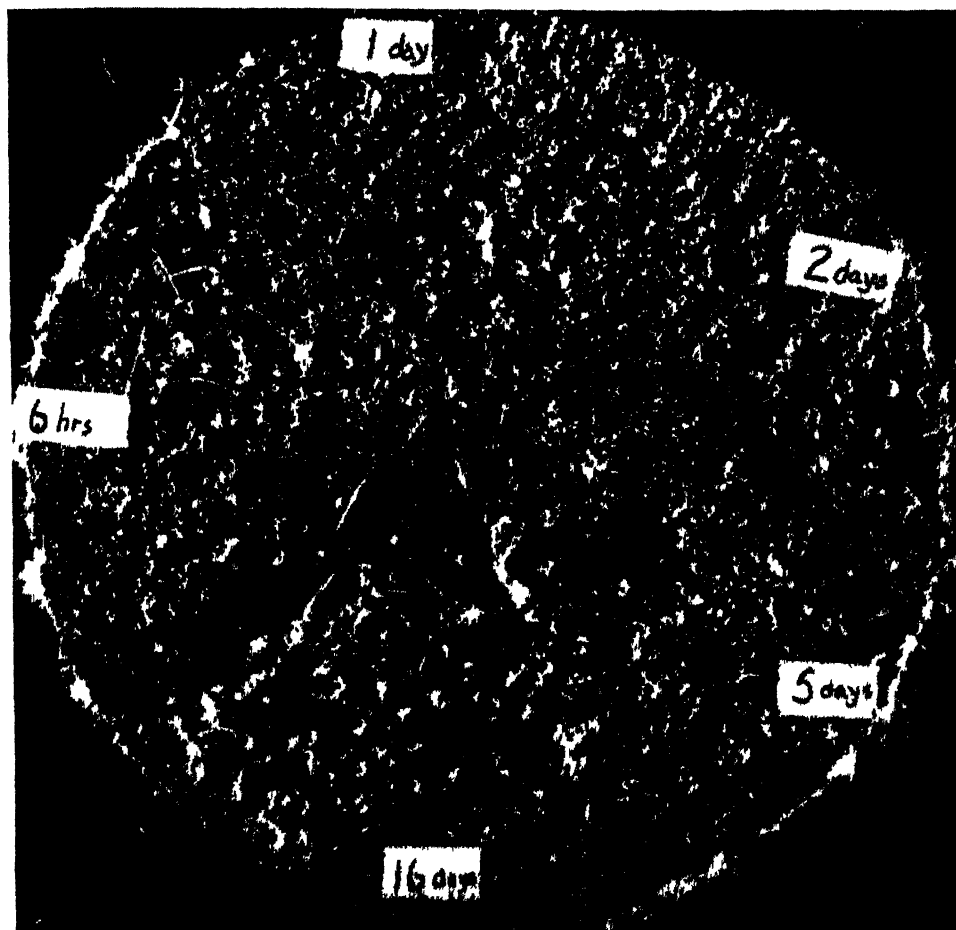


FIGURE 5. Same disc as in Figure 4 after further period of 19 days stationary immersion. Control to disc shown in Figure 6.

Examination of the discs at the conclusion of the test showed that while increasing current velocities diminished the growth rate and finally brought about loss of attachment, the weaker currents appeared to enhance growth. Thus, when rotated on Sector I, where growth had continued for 6 hours before rotation, *B. amphitrite*

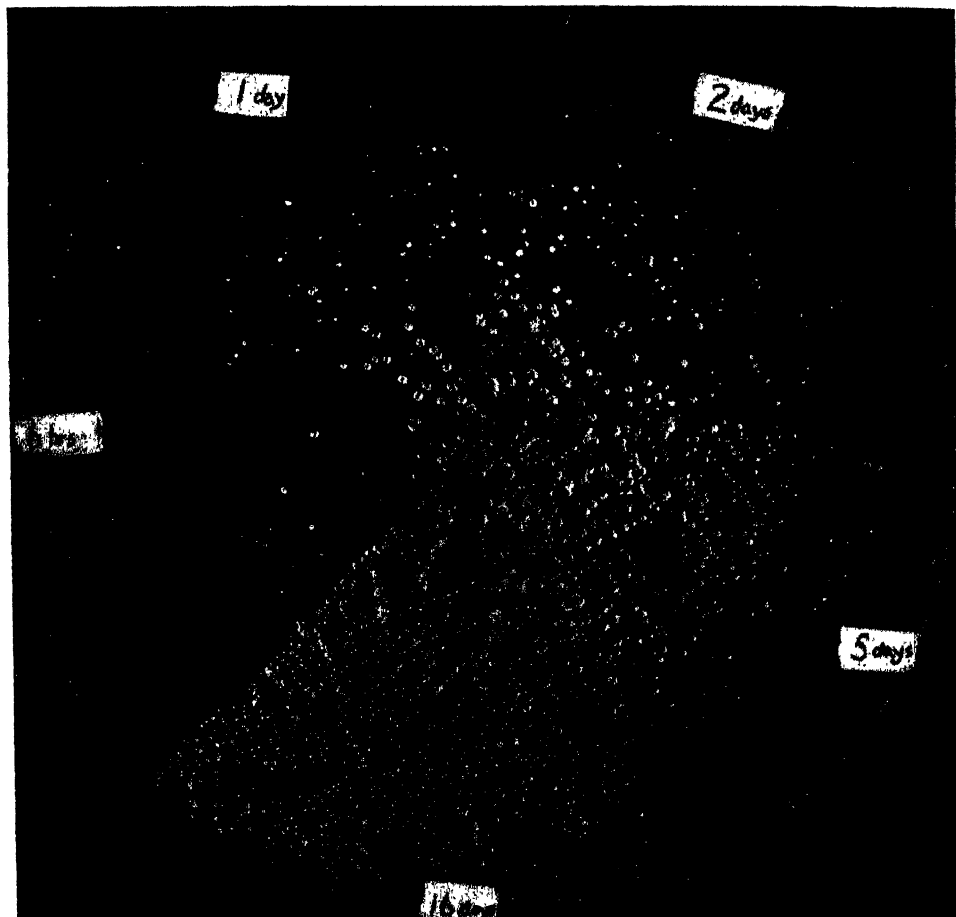


FIGURE 6. Similar disc to that of Figure 4 after period of 19 days rotation at 60 r.p.m. Age of barnacles before rotation is marked on each sector.

neither continued development nor remained attached at diameters beyond 16 inches, corresponding to a current of 2.7 knots (Table III). Within Sector VI, however, with barnacles originally 16 days old, a small number remained attached even at the outer edge of the disc, where the water flow approximated 4.7 knots. On the intervening sectors with barnacles of intermediate ages, the current velocities bringing about complete loss of attachment showed values between these extremes. Probably because of further attachments on the stationary control, the density of attachments on all sectors of the rotated disc was below that of the control.

The effects upon growth rate are illustrated by the observations in Table IV. On Sector I, within a 4 inch diameter, equivalent to 0.7 knot, barnacles reached a maximum width of 8 mm., compared with growth on the control of only 7 mm. At 2.3 knots, growth was reduced to only 3 mm. and at 2.7 knots, no barnacles re-

TABLE III

Effect of water currents upon adhesion of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by density of attachment in number per square inch. Experiment initiated January 8, 1944

Position on disc		Age before rotation				
Diameter (Inches)	Nominal water velocity (Knots)	Sector I (6 hours)	Sector II (1 day)	Sector III (2 days)	Sector IV (5 days)	Sector V (16 days)
0-28*	0	Dense	Dense	Dense	Dense	Dense
4	0.7	1.7	1.7	1.7	2.0	Too crowded to count
6	1.0	0	0.4	0.9	1.7	
8	1.3	0	0.4	1.0	1.3	
10	1.7	0.1	0.4	1.0	1.0	
12	2.0	0.2	0.4	0.6	1.9	
14	2.3	0.1	0.4	0.9	1.3	
16	2.7	0	0.4	0.6	0.9	
18	3.0	0	0.1	0.2	0.6	
20	3.3	0	0	0.1	0.4	
22	4.0	0	0	0	0	
28	4.7	Few in cracks	0	Few in cracks	Few in cracks	Few

* Stationary control.

mained on the sector. At intermediate current velocities conditions of growth lay between these two extremes, with normal growth occurring between 0.7 and 1.7 knots.

Similar observations were made upon barnacles of greater initial age within the remaining sectors. Thus, on Sector IV, where development had continued for a maximum period of 5 days before rotation, barnacles showed enhanced growth rate in currents up to one knot, normal growth similar to that of the control up to 2 knots, and almost complete cessation of growth at 3.3 knots. No barnacles remained on this sector at 4 knots.

Similar conditions of enhanced growth at low velocities, normal growth at intermediate velocities and cessation of growth with loss of attachment at higher velocities were observed on the remaining sectors, with barnacles of different initial ages.

The anomalous absence of enhanced growth on Sector V is probably due to the crowded condition of the barnacles which had become attached during a 16 day stationary period. The one day old barnacles were apparently less affected by the

TABLE IV

Effect of water currents upon growth of barnacles of different ages when subjected to 19 days rotation upon a submerged disc, as shown by diameter of largest barnacle in millimeters. Experiment initiated January 8, 1944

Position on disc		Age and size before rotation				
Diameter (Inches)	Nominal water velocity (Knots)	Sector I 6 hours (< 1 mm)	Sector II 1 day (< 1 mm)	Sector III 2 days (< 1 mm)	Sector IV 5 days (2 mm)	Sector V 16 days (5 mm)
0-28*	0	7 mm.	7 mm.	8 mm.	8 mm.	9 mm.
4	0.7	8 mm.	8 mm.	8 mm.	9 mm.	None more than 8 mm.
6	1.0	—	8 mm.	9 mm.	9 mm.	
8	1.3	—	8 mm.	8 mm.	8 mm.	
10	1.7	6 mm.	7 mm.	6 mm.	8 mm.	
12	2.0	5 mm.	5 mm.	6 mm.	8 mm.	
14	2.3	3 mm.	6 mm.	4 mm.	6 mm.	
16	2.7	—	5 mm.	3 mm.	5 mm.	
18	3.0	—	1.5 mm.	1.5 mm.	4 mm.	7 mm.
20	3.3	—	—	1.5 mm.	2.5 mm.	6 mm.
24	4.0	—	—	—	—	6 mm.
28	4.7	1.5 mm.	—	1.5 mm.	4 mm.	5 mm.

* Stationary control.

inhibitory action of water currents than the two day old barnacles. Otherwise, the adverse effects of water currents increased with their velocity, and decreased with the initial age of the barnacles.

On all sectors a few barnacles continued to grow at the perimeter of the disc where the presence of cracks provided local flow pockets with relatively still water.

The first experiment demonstrated an actual loss of barnacles at current velocities having critical values for the different initial ages of barnacles. In order to determine the time required for the loss of attachment to occur, a second experiment was carried out in which the disc was examined, not only at the beginning and end of the rotation period, but also at intervening times. The density of attachment and maximum size of barnacles were observed at each examination, as in the previous experiment.

The experiment was carried out during a period of heavy barnacle set. At the end of the stationary period, extending from February 19 to March 1, 1945, the density of attachments upon each sector, and particularly upon the sectors longest exposed, was very much greater than in the previous experiment.

TABLE V

*Density of barnacle attachment compared with rate of flow of water relative to the surface of a rotating disc, expressed in number per square inch **
(Figures in brackets indicate density upon a stationary panel serving as control; D, over 75 per square inch)

Stationary growth period	Diameter of disc (Inches)	Velocity (Knots)	Period of rotation							
			0 hrs.	1 hr.	12 hrs.	3 days	5 days	7 days	9 days	11 days
Sector I	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	25	25	20	15	10	8	5
	8	3	30	30	20	20	15	10	8	6
	12	5	30	30	20	20	15	6	6	6
	16	6.5	30	30	20	20	15	6	6	5
	20	8	30	30	20	20	15	4	4	2
6 hrs.	24	10	35	30	20	20	12	4	4	2
Sector II	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	30	30	30	20	15	15	8
	8	3	30	30	30	30	20	15	15	12
	12	5	30	30	30	30	20	15	15	6
	16	1.5	30	30	30	30	20	15	15	6
	20	8	30	30	30	30	20	10	10	5
12 hrs.	24	10	30	30	30	10	7	7	7	4
Sector III	Control	(0)	(30)	(30)	(30)	(30)	(30)	(60)	(D)	(D)
	4	1.5	30	30	30	20	20	12	12	10
	8	3	30	30	30	20	20	12	12	12
	12	5	30	30	30	20	12	10	10	10
	16	6.5	30	30	30	20	12	10	10	6
	20	8	30	30	30	20	12	7	7	5
1 day	24	10	30	30	30	7	5	5	4	4
Sector IV	Control	(0)	(30)	(30)	(30)	(40)	(40)	(50)	(D)	(D)
	4	1.5	30	30	30	20	20	17	17	15
	8	3	30	30	30	24	20	20	20	19
	12	5	30	30	30	24	20	20	15	9
	16	6.5	30	30	30	24	20	20	15	8
	20	8	30	30	30	15	10	10	10	7
2 days	24	10	30	30	30	10	7	7	6	6
Sector V	Control	(0)	(30)	(30)	(30)	(50)	(50)	(D)	(D)	(D)
	4	1.5	35	35	35	32	32	32	30	30
	8	3	35	35	30	30	30	30	30	30
	12	5	35	35	30	30	30	30	30	24
	16	6.5	35	35	30	30	30	30	30	15
	20	8	35	35	30	30	30	30	30	15
5 days	24	10	35	35	25	25	25	25	25	15
Sector VI	Control	(0)	(70)	(70)	(70)	(70)	(D)	(D)	(D)	(D)
	4	1.5	75	75	75	75	75	75	75	50
	8	3	75	75	75	70	70	70	70	30
	12	5	75	75	75	70	70	70	70	30
	16	6.5	75	75	75	70	70	70	70	25
	20	8	75	75	75	70	70	70	70	20
10 days	24	10	75	75	75	70	70	70	70	20

* Experiment initiated 2/19/45.

Observations showed that, as in the previous experiment, losses in attached barnacles occurred roughly in proportion to the current velocity, although at no point of the disc was the entire set lost. Similarly, the limiting rate of flow necessary for inhibition of growth or for loss of attachment increased generally with the initial age of the barnacles. Since observations were made at intervals during the experiment it was also possible to note the period of rotation necessary to bring about the first losses in attachment. This also increased generally with increasing age of initial growth. The observations are set forth in detail in Tables V and VI and summarized in Table VII.

In Table V the number of barnacles per square inch is recorded for various distances from the center of the disc, and for each individual sector. These observations are repeated for various intervals of time during the experiment.

An increase took place in numbers on the control panels due to continued attachment of cyprids, whereas on the rotated disc barnacles became progressively reduced in number as the experiment continued. After one hour of rotation the only losses were among the six hour old barnacles on Sector I. These losses were very small, however, and although observed at current velocities of 1.5 knots and 10 knots, they did not occur at intervening velocities. The first significant losses took place twelve hours after the start of rotation among barnacles less than twelve hours old. Of the older ones, only those 5 days old showed losses, amounting to a change at most velocities from 35 to 30 per unit area.

Barnacles on the remaining sectors, varying in age from twelve hours upwards, were not observed to diminish in number until the third day of rotation. The smallest loss over this period took place among the twelve hour barnacles, which were only lost at speeds of ten knots, whereas, after a similar period of rotation, other barnacles of greater ages had been lost even at 1.5 knot current velocity. Ten day old barnacles were not greatly disturbed by 1.5 knot currents until after eleven days' rotation. These observations, summarized in Table VII, indicate that in general the velocity of current necessary to dislodge barnacles increased with the age they had reached before the experiment. Barnacles twelve hours old, however, showed a marked increase in resistance to being dislodged, even compared to those ten days old, whereas those two days old, on the other hand, seemed to be less resistant than younger or older ones. It is also noteworthy that in general the duration of rotation necessary to bring about the first losses depends much more upon the initial age of the barnacle than upon the current velocities.

In a similar manner the percentage of the original number of barnacles remaining following an eleven day period of rotation increased with increasing age before rotation. The ten day old barnacles at the end of the experiment, however, showed poorer resistance and had a lower percentage of continued attachment than in the case of the five day barnacles. It must be noted, however, that the ten day barnacles were densely crowded and it is possible that this factor decreased their resistance to dislodgement.

Further examination of the results in Table V discloses that losses continued to occur to some extent on all sectors at the end of eleven days, although they were less on those sectors where initial age of the barnacle was greater.

Observations of the size of barnacles recorded during the experiment are given in Table VI. On Sector I the six hour old barnacles within a diameter of 8 inches, equivalent to a three knot water current, continued to grow, although at a slow rate.

TABLE VI

*Growth of barnacles in relation to flow of water over the surface of a rotating disc,
expressed as greatest diameter in millimeters *
(Figures in brackets refer to stationary control)*

Stationary growth period	Diameter of disc (Inches)	Velocity (Knots)	Period of rotation							
			0 hrs.	1 hr.	12 hrs.	3 days	5 days	7 days	9 days	11 days
Sector I	Control	(0)	(0.3)	(0.3)	(0.3)	(0.5)	(0.8)	(1.3)	(2.5)	(3.0)
	4	1.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.5
	8	3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	12	5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	16	6.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	20	8	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	24	10	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sector II	Control	(0)	(0.3)	(0.3)	(0.3)	(0.6)	(0.8)	(2.5)	(3.0)	(3.5)
	4	1.5	0.3	0.3	0.3	0.3	0.3	0.3	1.0	1.5
	8	3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	12	5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	16	6.5	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	20	8	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	24	10	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Sector III	Control	(0)	(0.3)	(0.3)	(0.3)	(0.6)	(0.8)	(2.0)	(3.0)	(3.5)
	4	1.5	0.3	0.3	0.3	0.5	0.5	1.0	2.0	3.0
	8	3	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	12	5	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	16	6.5	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	20	8	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
	24	10	0.3	0.3	0.3	0.5	0.5	0.5	0.5	0.5
Sector IV	Control	(0)	(0.3)	(0.3)	(0.5)	(0.8)	(1.0)	(3.0)	(3.5)	(4.0)
	4	1.5	0.3	0.3	0.5	1.0	1.0	1.3	1.3	1.5
	8	3	0.3	0.3	0.3	0.8	0.8	0.8	0.8	1.0
	12	5	0.3	0.3	0.3	0.8	0.8	0.8	0.8	1.0
	16	6.5	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
	20	8	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
	24	10	0.3	0.3	0.3	0.3	0.8	0.8	0.8	0.8
Sector V	Control	(0)	(0.8)	(1.0)	(1.5)	(2.5)	(3.0)	(4.0)	(5.0)	(6.0)
	4	1.5	0.5	0.8	1.0	2.5	2.5	3.0	3.0	3.5
	8	3	0.5	0.8	0.8	1.5	1.5	1.5	1.5	1.5
	12	5	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	16	6.5	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	20	8	0.5	0.5	0.8	1.5	1.5	1.0	1.5	1.5
	24	10	0.5	0.5	0.8	0.8	0.8	0.8	0.8	0.8
Sector VI	Control	(0)	(2.5)	(2.0)	(2.5)	(4.0)	(5.0)	(7.0)	(9.0)	(9.0)
	4	1.5	2.0	2.0	2.0	3.5	3.5	4.5	4.5	5.0
	8	3	2.0	2.0	2.5	3.0	3.0	3.5	3.5	3.5
	12	5	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	16	6.5	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	20	8	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5
	24	10	2.0	2.0	2.0	3.0	3.0	3.5	3.5	3.5

* Experiment initiated 2/19/45.

TABLE VII

*Summary of observations on the effect of water currents produced by rotation upon attached barnacles. Expressed as multiples of normal growth, period before loss in number first occurred, and percentage remaining at the end of eleven days **

Current velocity (Knots)	Period of stationary growth					
	6 Hrs.	12 Hrs.	1 Day	2 Days	5 Days	10 Days
1.5	0.1 × (1 Hour) 20%	0.4 × 5 Days 30%	0.8 × 3 Days 30%	0.3 × 3 Days 50%	0.6 × 3 Days 90%	0.6 × 11 Days 70%
3	Inhibition 12 Hours 30%	Inhibition 5 Days 40%	Inhibition 3 Days 40%	0.3 × 3 Days 60%	0.2 × 2 Days 90%	0.3 × 3 Days 40%
5	— 12 Hours 20%	— 5 Days 20%	— 3 Days 30%	0.1 × 3 Days 30%	0.2 × 2 Days 70%	0.3 × 3 Days 40%
6.5	— 12 Hours 20%	— 5 Days 20%	— 3 Days 20%	Inhibition 3 Days 30%	0.2 × 2 Days 60%	0.3 × 3 Days 30%
8	— 12 Hours 20%	— 5 Days 20%	— 3 Days 20%	— 3 Days 20%	Inhibition 2 Days 60%	0.3 × 3 Days 30%
10	— (1 Hour) 10%	— 3 Days 20%	— 3 Days 20%	— 3 Days 20%	— 2 Days 60%	0.3 × 3 Days 30%

* Experiment initiated 2/19/45.

Even at velocities below 1.5 knots the growth was considerably less than that of the stationary control. The ten day old barnacles on Sector VI continued to grow near the circumference, although subjected to ten knot currents. Barnacles of intermediate age showed a reduction of growth inversely dependent upon their initial age, and directly dependent upon the current. Barnacles less than two days old ceased growth at water currents over three knots, and only the ten day old barnacles continued growth at ten knots.

At the end of this experiment the remaining barnacles were examined to determine whether any considerable portion remaining attached was dead. A few of the older barnacles had lost their soft parts, but it was difficult to obtain a reliable criterion as to the condition of the remainder. It was not definitely established, therefore, what proportion had died.

Differences between the results of this and the previous experiment lie mainly in the higher current velocities required to bring about growth inhibition and loss of attachment and the absence of complete loss at any part of the disc in the later experiment. This apparent anomaly may be partly due to the much greater density of initial attachment, which may have reduced the *effective* velocity of the currents by providing a much more irregular surface. It is also conceivable that had the

experiment continued for the full 19 day period of the first experiment rather than 11 days only, the growth inhibition effect might have appeared at lower velocities and might have resulted in complete loss at some parts of the disc.

DISCUSSION

The experimental results indicated that current velocities limiting the attachment of barnacles lie in the neighborhood of one knot and that the limits for the three species, *Balanus eburneus*, *B. amphitrite*, and *B. improvisus* are in ascending order of magnitude. That a one knot current should limit attachment is in accordance with the conclusions of Visscher and earlier workers that attachment of barnacles does not readily take place upon ships in motion.

It further appears that growth rates of previously attached barnacles are reduced by water currents in inverse proportion to the age of the barnacle and directly in proportion to the current velocity. The effect of crowding together is further to reduce the growth rate. The effect of very low current velocities is to increase the rate of growth. A slight loss of attachment occurs at all current velocities, but this may be partly due to overcrowding. Large and significant losses take place at velocities which are sufficient to reduce the growth rate. These losses begin some hours after the start of rotation, and continue for a period of at least eleven days.

Since losses due to dislodgement do not occur during the first few hours of rotation, except in the case of barnacles attached within six hours or less, and since they continue to take place during the entire period of rotation, it seems probable that they are not directly due to mechanical action. Possibly the mechanism is one of interference with feeding processes, followed by reduced growth rate, death and diminished adhesion.

A further point arising out of the experimental data is the fact that at equivalent current velocities 12 hour and 24 hour old barnacles show greater growth and less loss of attachment than those somewhat older. It is possible that at this stage of development, with metamorphosis incomplete, an orientation to the current may occur which facilitates feeding, although this was not actually observed.

Once attached the barnacles are able to withstand current velocities of increasing magnitude, although for the first two or three weeks the growth rate is considerably reduced. Loss of attachment appears to follow reduced growth rate and may involve actual death from lack of food. The current velocities required to bring this about are sufficiently low to provide support for Visscher's conclusion that barnacles are killed by ocean passages of 500 miles or more, if vessels of more than 10 knots performance are considered, or if the age of attached barnacles is no greater than a 10 day stay in port would allow. On the other hand, current velocities lying below this figure, such as would be produced by slow sailing vessels, would appear from the experimental data to be insufficient to bring about a loss of the attached barnacles. In the case of faster ships, stays in port of longer than a few days might also permit the accumulated barnacles to survive an ocean passage. This type of ship service would readily provide a means of distribution for barnacles. It would explain Fischer-Piette's observations of the appearance of *Balanus amphitrite* on Mediterranean shores, and would support his hypothesis of distribution by ships.

The enhanced growth rate in current velocities of 1.3 knots and less, depending on the age of the barnacle, provides a quantitative expression for the observations

of Rice, Moore, Kitching, and others upon the increased growth of barnacles subjected to water currents.

The conditions in a harbor or an embayed or open coast are rarely such that tidal currents in excess of one knot are continuously present. Under these conditions, therefore, there would be no obstacle to the attachment of barnacles. Once attached, however, the barnacles would usually be subjected to intermittent tidal currents.

No data are available as to the effects of intermittent currents but it would be reasonable to assume that these would be, if anything, less than the effects of continuous currents. The data presented here would therefore make it appear unlikely that any but the strongest of tidal currents would prevent the attachment and continued development of barnacles on rocks or stone, and wooden piling. Where fairly continuous currents of less than 1.5 knots were present the growth would be encouraged.

A different situation occurs in estuaries where continuous currents might occur. Here, however, the limiting factor, according to the work of previous authors, is probably lowered salinity, since continuous currents would scarcely permit of the estuary remaining salt.

Where wave action is strong the effects might be considered as equivalent to strong currents of frequently changing direction, which might conceivably inhibit attachment or growth, even below tide levels. Even where attachment occurred during temporary lulls, the strong recurrent wave action might be expected to dislodge the barnacles. This may explain the observations of Stephenson and his co-workers that some species of barnacles do not occur in a zone of heavy wave action.

The effect of water currents under natural conditions may therefore be summarized as one of enhanced growth with velocities below 1.5 knots, and one of reduced growth above this. It would rarely happen under natural conditions that water currents alone would prevent barnacle colonization. Strong wave action, however, might be expected to have this effect.

Certain qualifications should be added to the above discussion which may explain some anomalous observations. The arguments above are based on the calculated rates of flow of water over the rotating disc, and the results of experiments have indicated that these rates are slightly higher than the actual rates of flow taking place. The error, however, does not appear to be greater than 10 per cent to 20 per cent, and does not qualitatively affect the validity of the general conclusions.

Since the observations were made upon the relatively smooth surface of a wooden disc, they would not be applicable to very rough surfaces where local pockets of relatively still water might develop, to the areas immediately adjacent to lapped plates of ships' hulls, or to portions of piling or other submerged surfaces where the contours and configuration might produce local stagnation effects.

SUMMARY

1. The work of previous authors, dealing with the effect of water currents upon barnacle attachment, growth and distribution is briefly reviewed.

2. Experiments were conducted to determine the effects of water currents upon the attachment and growth of barnacles, and particularly of *Balanus amphitrite*.

Submerged rotating discs and glass tubes of graded cross-sectional diameter were employed to provide variations in current velocity.

3. The velocity of water current limiting attachment appears to lie between 0.5 and 0.9 knot for *Balanus amphitrite*, between 0.4 and 0.7 knot for *B. eburneus*, and above 1.1 knots for *B. improvisus*.

4. Following attachment the growth rate of barnacles was found to be increased by water currents of velocity less than 1.5 knots and to be decreased by currents with velocities in excess of this. The adverse effects of water currents were found to decrease with increasing age of the barnacles subsequent to attachment. Six hours after attachment, growth rate was reduced to one-third of normal by a 1.5 knot current and completely stopped by a 3 knot current. Five days after attachment, growth was prevented by currents ranging between 3.3 and 8 knots.

Loss of attachment appeared to some extent among all barnacles in which growth rate was reduced. This loss was greatest at velocities bringing about complete cessation of growth.

5. It is suggested that loss of attachment is due to interference with the feeding process, followed by reduction of growth rate, death, and diminished adherence. Possibly due to an orientation to the current which facilitates feeding, barnacles attached for one day or less show less retardation of growth rate and loss of adherence than barnacles two days old.

6. Since tidal currents are almost invariably intermittent it appears from the data presented that they are not sufficient to prevent the colonization of suitable surfaces by barnacles, except where the velocities are unusually high.

It also follows from the numerical results obtained that on vessels making short stays in port and relatively long voyages, little permanent barnacle fouling will occur, since those organisms which attach will be killed and at least a portion of them dislodged. The evidence does not preclude, however, the continued growth of barnacles upon slow vessels making longer stays in port, and their geographical distribution by this means.

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MICRURGICAL STUDIES ON CHIRONOMUS SALIVARY GLAND CHROMOSOMES¹

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Particular interest in the giant salivary gland chromosomes of dipteran larvae was awakened by Painter (1933) and Heitz and Bauer (1933) who demonstrated the existence of a close relationship between the genetic significance of the chromosomes and their structure. Regarding the gross structure of these chromosomes there is general agreement, but not for fine details. Bauer (1936) and Painter and Griffen (1937) offered cytological evidence for a polytene concept of chromosome structure from observation, in fixed and stained preparations, of numerous longitudinal striations which they considered to be chromonemata. Metz and Lawrence (1937) and Metz (1941) maintained that the longitudinal striations appearing in these fixed and stained preparations were merely the result of drawing out the walls of alveoli. Buck (1942) afforded support for this view by showing in osmic vapor-treated chromosomes that an original alveolar structure could be reversibly transformed into a fibrillar-like one by micromanipulation. Kodani (1942), using cytolytic methods with alkali and urea, found evidence for only four chromonemata in each chromosome, a condition which Painter and Griffen (1937) and Buck (1937) observed in the early larval stages, but not in the giant chromosomes of later stages. More recently, Frolova (1944) has given experimental support to the polytene concept by demonstrating the presence of numerous longitudinal strands in fixed chromosomes after partial digestion with nuclease preparations.

Aside from their cytogenetic interest, the salivary gland chromosomes, because of their large size and visibility *in vivo*, make especially suitable material for studies on physical properties. Using the micrurgical technique, various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) have found the freshly isolated salivary gland chromosomes to be tough, viscid, elastic gels. These observations are in essential agreement with those first made by Chambers and Sands (1923) on the chromosomes of *Tradescantia* pollen mother cells and by Chambers (1924a) on those of *Dissosteria* spermatocytes. Buck (1942), using osmic vapor-treated chromosomes, reported more detailed findings on elasticity. That the physical properties of the chromosomes may be easily modified by the presence of torn cytoplasm or calcium ions was shown by Duryee (1941) in his studies on amphibian chromosomes. The observations of Chambers (1924b) that torn cytoplasm is acidic, and of Chambers and Reznikoff (1926) that the micro-injection of various salts into protoplasm may have detrimental effects,

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further point out the need for precaution in studying the physical properties and structure of the chromosomes.

This investigation is concerned with the structure and physical properties of the salivary gland chromosomes as observed within the intact fresh cell and after isolation into media designed to maintain them in the fresh condition.

Grateful acknowledgment is made to Prof. Robert Chambers, under whose guidance this work was done, for his constant aid and invaluable criticism.

MATERIALS AND METHODS

The species selected for study were *Chironomus plumosus*, *C. tentans*, and certain smaller unidentified species of *Chironomus*. *Chironomus* larvae were chosen in preference to those of other Diptera because of their larger chromosomes (maximum size, 20 μ in diameter and 150 μ long) and the flattened shape of the salivary gland, factors which facilitate micromanipulation. Moreover, the chromosomes were always plainly visible in the living cell, the bands being especially well defined. Incidental observations were made on *Drosophila* chromosomes and they were found to agree in all important respects.

The glands were carefully removed from mature larvae under a dissecting microscope and immediately immersed in either a drop of *Chironomus* hemolymph or amphibian Ringer's solution which was found to be isotonic. Care was taken to avoid the presence of any tissue but the gland itself since otherwise the medium became unduly acid. The hemolymph was obtained by inserting a pipette through the body wall of a larva into the hemocoel. In order to be satisfactory the hemolymph had to be freshly obtained and immediately covered with paraffin oil to prevent the development of alkalinity which occurred on exposure to air.

The micromanipulation was carried out immediately after mounting a single intact gland in the medium on a thoroughly cleaned coverslip which was then inverted over the moist chamber of a Chambers micromanipulator. Structural changes within the chromosomes of the intact cells do not appear for many hours in hemolymph or Ringer's solution, but extra precautions were taken by making observations within 15 minutes after mounting the glands. The micropipettes routinely used had an inside bore of never over one μ , and the microneedles used for fine dissection of the chromosomes tapered rapidly to points approaching the limit of visibility.

Nuclei and chromosomes were removed from disintegrating cytoplasm as quickly as possible. This was necessary in order to preserve their normal appearance, an observation in agreement with that of Duryee (1937) on amphibian germinal vesicles. To remove the chromosomes, an entire gland was mounted under a dissecting microscope in a medium, to be described later, in which the chromosomes were to be isolated. By means of fine steel needles a cell was quickly torn and the nucleus ruptured. The torn nucleus remained in situ, and, by exerting a gentle pressure on the torn cell, the chromosomes could be forced out of the nucleus and floated away without coming in contact with the injured cytoplasm. The loosely adhering clump of chromosomes was immediately transferred by means of a lip pipette to a fresh drop of the medium to be used, then mounted in the usual manner in the moist chamber. In this way the chromosomes were found to retain most nearly their normal appearance and physical properties after isolation, their sub-

sequent condition depending on the medium in which they remained. No medium was found which would maintain the chromosomes in their normal condition indefinitely nor were extensive attempts made to find such a medium. Among the media tried and found to be unsatisfactory were hemolymph, Ringer's solution, paraffin oil, 0.1 M NaCl-0.01 M KCl solution used by Duryee (1941), 0.25 per cent egg albumen solution used by Melland (1938), and 0.3 M sucrose solution suggested by the work of Chambers and Sands (1933). These special solutions were used without regard to pH conditions.

From determinations on a great variety of cells including the *Chironomus* salivary gland nucleus, Chambers (1929) found the intranuclear pH to be within 7.6-7.8. It would be expected therefore that the medium in which the isolation of the chromosomes is to be done should have a pH within the aforementioned limits. However, the most satisfactory mixture, when buffered to pH 7.6-7.8, caused the isolated chromosomes to swell and become excessively sticky. Moreover, the swelling was accompanied by a fading of internal structure. However, at pH 7.0, the chromosomes closely approached, both in appearance and physical properties, those within the nucleus. It should be noted that the consistency was slightly greater than that of the intact chromosomes. The structure and properties of the chromosomes were maintained throughout the experimental period. The medium used consisted of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer, pH 7.0.

Observations and micromanipulation experiments were routinely made with a Leitz microscope using a 1.8 mm. oil immersion objective, N.A. 1.25, and a 10 × ocular. A micrometer ocular was used for measurements.

OBSERVATIONS

The chromosomes in the fresh cell in Ringer's solution or hemolymph

The chromosomes and nucleolus in the fresh cell were always plainly visible, the bands being sharply defined and either beaded or homogeneous in appearance. A hyaline material, variously referred to as nuclear sap, nucleoplasm, or karyolymph, separated the chromosomes from each other. It has been shown (Glancy, 1940) that this hyaline material is differentiated into a central portion of jelly-like consistency in which the chromosomes are embedded, and a more fluid peripheral zone. The evidence for this regional differentiation was based on experiments involving the injecting of oil drops and carbon particles as well as the manipulation of the chromosomes. The hyaline material is referred to in this paper as the nuclear matrix.

Stickiness. It was found possible to insert microneedles into the nucleus and to force the chromosomes against each other as well as against other structures in the nucleus, and also to push them against injected oil drops and carbon particles. Two chromosomes pushed together by means of microneedles could easily be separated again without sticking to each other. It should be noted, however, that a narrow hyaline zone, presumably the jellied nuclear matrix, always remained between them, thus probably preventing actual contact of the two chromosomes. Similar results were obtained when a chromosome was forced against the nucleolus or the nuclear membrane.

A micropipette containing an aqueous carbon suspension was inserted as close as possible to a chromosome without actually touching it, and a small quantity of the suspension injected. In none of the attempts were carbon particles seen to adhere to the chromosomes, nor did they ever make contact with a chromosome. However, injection of a large amount of fluid caused liquefaction of the jelly matrix, thus permitting the carbon particles to make contact with the chromosomes. In a similar manner, various oils; namely, Nujol, olive, almond, and peanut oils, were injected into the nucleus. None of the oil drops adhered to the chromosomes.

When the cytoplasm was torn without tearing the nucleus, a change occurred in the nucleus. This was made evident by the marked shrinkage of the chromosomes. Two chromosomes pushed together in the nucleus of such a torn cell invariably stuck to each other, and thick viscid strands were pulled out when attempts were made to separate them. The chromosomes also adhered to the nucleolus, the nuclear membrane, injected oil drops, carbon particles, and the microneedles. In every case, separation could be achieved only when accompanied by thick viscid strands pulled out from the chromosomes.

In summary, these experiments show that the chromosomes, because they are prevented from coming in contact by the jelly-like nuclear matrix, appear to be non-sticky in the intact cell. Liquefaction of this matrix occurs when the cytoplasm is torn, thus permitting the chromosomes to make contact. They are then found to be sticky.

Consistency. The micrurgical experiments to be described indicate that, compared to the cytoplasm and the nuclear matrix, the chromosomes are relatively soft, easily deformable gels, and that there are differences in consistency between the bands and the interband regions.

A very finely tapered microneedle was pushed up through the cytoplasm and through the nuclear membrane, into the interior of the nucleus. The needle was pushed in from the side, and even before it was brought into actual contact with a chromosome, the surface of the chromosome became indented on the side next to the needle. The needle was pushed still farther and when its tip went into the interior of the chromosome, the indentation disappeared and the original contour of the chromosome was restored. This deformation of the chromosome must be due to the central jelly-like matrix since insertion of the needle into the more fluid peripheral region of the nucleus had no such deforming action on the chromosomes. The indentation of the chromosome, when the needle tip was being brought into its vicinity, and the readiness with which the chromosome returned to its original shape after the needle had penetrated indicated that the chromosome was of a softer consistency than the surrounding central nuclear matrix.

The relatively low consistency of the chromosomes compared to the central nuclear matrix was also shown by experiments in which oil drops were injected into the nucleus. The effect of the oil drops in causing deformation of an adjacent chromosome differed according to the site of injection. Drops of Nujol, olive, almond, and peanut oils, 1-20 μ in diameter, were injected close to a chromosome. Large oil drops carefully injected into the central jelly-like nuclear matrix produced a rounded depression in the nearest chromosome. However, this did not occur when the matrix was agitated by the needle, thus showing the thixotropic nature of the jelly matrix. Peripherally injected oil drops were never observed to cause de-

formation. These results indicate that the consistency of the central nuclear matrix is greater than that of the chromosomes, whereas the reverse is true in the peripheral region of the nucleus.

The nucleolus was found to be the most easily deformed body in the nucleus. This was shown by forcing the nucleolus against the nuclear membrane, the chromosomes, and the microneedles. In all cases the nucleolus was deformed in the manner of a fluid drop. When the nucleolus was separated by a microneedle from the small fourth chromosome to which it is normally attached in *Chironomus*, the nucleolus became spherical.

Evidence for differences in consistency between the bands and the interband regions was obtained in several ways. A microneedle inserted into a chromosome and then withdrawn produced a space which closed over more rapidly in an interband region than in a band. The thin bands were intermediate between the interband regions and the thick bands in this respect. The longer persistence of the hole in the bands is indicative of a higher consistency. The greater resistance of the bands, especially the thicker ones, to deformation during manipulation of the chromosomes, as in the stretching experiments, also is in accord with their higher consistency. The results of injecting oil drops into the chromosomes led to similar conclusions. Of the various oils used, Nujol, olive, almond, and peanut oils, only the last two could be readily injected into a chromosome. This was easily done in an interband region, but only with difficulty or not at all in a band. The injected oil drops became spherical, showing the relatively low consistency of the interband regions, and caused the displacement of the relatively viscid bands on either side.

Extensibility and elasticity. Observations on extensibility and elasticity were made by stretching a small portion of a chromosome between two microneedles. The degree of stretching which could be performed, however, was limited since the nuclear membrane was torn when the microneedles were moved more than approximately 20–30 μ apart. In all cases in which a part of a chromosome was stretched within the nucleus, release from the needles resulted in a complete return to the original appearance.

A short region of a chromosome was seized lengthwise between two microneedles and the needles were slowly drawn apart. As the chromosome was stretched, there was a decrease in its diameter, and its structure became less distinct. The thick bands were more resistant to stretching than the thinner ones, whereas the interband regions stretched most readily. During the stretching the broad bands often separated into several narrow bands, thus demonstrating their compound nature. Faint longitudinal striations which followed the lines of tension generally appeared in the interband regions when a chromosome was stretched more than twice its length. These stress lines became more pronounced as stretching was increased. They did not appear to be identical with the delicate longitudinal striations occasionally seen in unstretched fresh chromosomes. On release of tension, the striations disappeared and the chromosome, even after being stretched five times its length, returned to its original condition. It was not found possible to stretch a given region more than this without tearing the nuclear membrane. In every case, the chromosomes recovered completely after the microneedles were removed.

Lateral stretching of the chromosomes was also performed. Two microneedles were inserted into a chromosome close to the margins, then slowly drawn apart.

The chromosome cylinder flattened during the process and bands which had appeared homogeneous became beaded (Fig. 1). As tension was increased, stress lines, radiating from the point of each microneedle to the chromosome, became visible. On release of tension these lines disappeared, and the beaded appearance of the bands was lost. A chromosome could be stretched approximately three times its original width and recover fully.

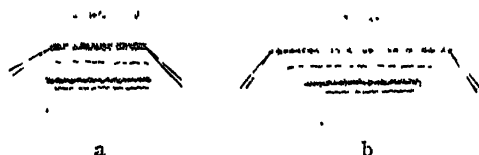


FIGURE 1a. Sketch of a portion of a chromosome showing two microneedles inserted directly into a band prior to lateral stretching of the chromosome within the intact cell. All sketches are of *Chironomus* chromosomes.

FIGURE 1b. Beaded appearance of the band when the chromosome in (a) is stretched laterally. Note tension lines in the interband regions.

The chromosome membrane. The micrurgical evidence to be presented indicates that the salivary chromosome possesses a delicate elastic membrane. A microneedle was inserted into the margin of a chromosome within the intact nucleus, in an attempt to remove any existing membrane. When the needle was slowly and carefully withdrawn, faint lines were seen radiating from it to the chromosome (Fig. 2a). To test whether or not these lines were actually folds in

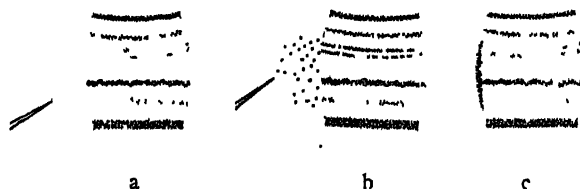


FIGURE 2a. Lifting by a microneedle of a membrane-like material from a portion of a chromosome within the intact cell.

FIGURE 2b. Micro-injection of a carbon suspension into the area subjacent to the lifted membrane shown in (a).

FIGURE 2c. Concentration of the carbon particles into a narrow zone as a result of gradual contraction of the membrane.

a membrane which had been lifted off from the chromosome the same operation was repeated and an aqueous carbon suspension was injected into the triangular area in question. The carbon particles converted the region showing the radiating lines into a sac-like structure which remained connected to the chromosome (Fig. 2b). When the micropipette was carefully withdrawn, the fluid evidently escaped from the area, as the sac slowly contracted and the carbon particles came closer to the chromosome where they became packed against its surface (Fig. 2c). Repetition of the

experiment injecting a larger quantity of the suspension into the triangular area first converted it into a sac; then as injection was continued, the sac burst, and the carbon particles scattered in the matrix where they briefly exhibited Brownian movement.

Oil drops were also injected in a similar manner into the triangular area produced by pulling on the margin of the chromosome. After the withdrawal of the micropipette, the oil was gradually drawn to the chromosome surface where it remained as a slight protuberance. Larger oil drops were injected and could be seen to be held against the chromosome by what appeared to be a membrane. No deformation of the chromosome occurred, presumably because there was no jelly-like matrix between the oil drop and the chromosome.

Various salt solutions (0.01–1.0 M NaCl, KCl, and CaCl_2) and also distilled water were injected into an interband region in an amount which caused a swelling of the region without breaking its contour. Figures 3a, b, and c show the condi-

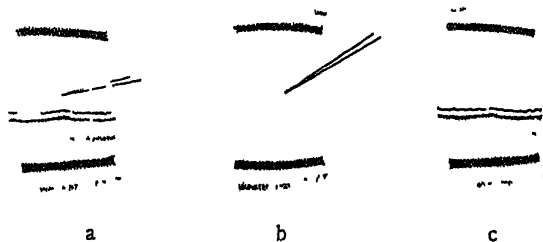


FIGURE 3a. Micropipette inserted into an interband region of a portion of a chromosome within the intact cell showing the initial stage of injection.

FIGURE 3b. Localized swelling and loss of structure in the injected portion of the chromosome. Results are similar with solutions of NaCl, KCl, CaCl_2 , and distilled water.

FIGURE 3c. The appearance of the same chromosome after injection of sufficient CaCl_2 solution to rupture the injected region. The bands have reappeared in their original positions, but the distinct boundary (membrane) shown in (a) is no longer evident. Note the granular region of the nuclear matrix.

tion of the chromosome before, during, and after the injection. With the progress of the injection, the lesser bands in the vicinity faded out and the zone underwent swelling until a heavy band on each side of the injected zone was reached. The injection was continued within the limit of bursting. The pipette was then removed, and the swollen zone returned to its original dimensions in which the final appearance was that of the region prior to injection. The surface of the injected region could be ruptured when the amount of fluid injected was sufficient to cause it to swell to approximately two and a half times its original diameter. Thereupon the membrane-like boundary suddenly disappeared irrespective of the fluid injected. When CaCl_2 was used a granulation appeared in the nuclear matrix around the injected zone (Fig. 3c). This did not occur with water, NaCl, or KCl solutions.

It may be concluded, therefore, that the salivary chromosome possesses a delicate elastic membrane.

Internal Structure. The visible structure of the chromosomes was studied by careful and extended observations of the chromosomes within the intact nucleus

both of the isolated gland and of glands within the intact larvae.² For this purpose the observations were made with a Leitz 1.4 N.A. apochromatic oil objective and a triple lens condenser. The equipment was sufficient to resolve the hexagonal apertures of the *Pleurosigma angulatum*. The distinct and sharply defined bands were beaded in some regions, and homogeneous in appearance in others. The interband regions, which were much less refractile, generally appeared either homogeneous or finely granular. In unusually large chromosomes the granulation was coarse and often the arrangement gave the effect of longitudinal striations. These granular striations, which were seen at all levels in the chromosomes, were individually connected to the beads in the bands. During these observations the chromosomes were not under tension and in the case of the isolated gland were free from compression.

That the salivary gland chromosome is made up of numerous longitudinal fibrils was further indicated by microdissection. The chromosome membrane was first removed by a very fine microneedle over a length of several bands from the region to be studied. The needle was then inserted into the margin of an exposed interband region. This adhered to the needle and, when the needle was carefully moved to one side, a strip of very thin fibril with one end on the tip of the needle could be seen being carried away from the chromosome. As the fibril was being carried still farther it was separated from the chromosome over a length of several bands (Fig. 4).

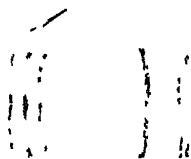


FIGURE 4. A single fibril with a chromonema-like appearance being removed by a microneedle from a chromosome within the intact cell.

The isolated fibril showed nodular swellings which corresponded in position with the bands from which it was being separated. This was done over and over again with the same chromosome and with different chromosomes. The same phenomenon could be observed whether the bands on the operated chromosome were homogeneous or beaded. The fibrils could be stretched and, when tension was released, they returned to their original lengths. One case was observed in which a fraying of the chromosome resulted when a pipette had been inserted to inject water. The injection was made close to the periphery and the pipette then removed. These partially isolated fibrils closely resembled that in Figure 4. The microdissection experiments were performed more readily with large chromosomes in which longitudinal striations were visible. They were also done with the chromosomes in which the interband regions appeared homogeneous. The dissection evidence strongly suggests that longitudinal fibrils possessing nodular swellings really exist in the chromosomes.

The state of the chromosomes in nuclei rendered optically homogeneous by alkaline or hypotonic Ringer's solution. The question has frequently been

² As Buck (1938) has already described, the intact larvae can be mounted between slide and coverslip and because of the transparency of its body wall, the cells in the salivary glands are easily visible under high power, and can be satisfactorily observed. Their appearance was identical with that of the isolated glands mounted in Ringer's solution.

raised whether chromosomes in so-called hyaline nuclei maintain their morphological integrity or whether they lose their identity through dispersion in the hyaline phase. Naturally occurring hyaline salivary gland nuclei were never found, but could be readily produced by immersion of the gland in slightly hypotonic or in mildly alkaline (pH 8.4) Ringer's solution. Within a few minutes after immersion the nucleus swelled, chromosomes faded, the interbands disappearing first, and finally only the nucleolus remained visible. When the gland was returned after several minutes to isotonic Ringer's solution at pH 7.0, the chromosomes reappeared.

A microneedle could be inserted into one of these hyaline nuclei without causing its collapse and usually without producing any visible effect. In some cases, however, the nuclear membrane wrinkled slightly when the microneedle was withdrawn, indicating that fluid was lost. When a microneedle was moved slowly back and forth within the nucleus no regional resistance to its movement was encountered. Similarly no resistance was offered to movement of the nucleolus throughout the entire nucleus. Return of the gland to Ringer's solution caused the chromosomes to reappear. It is noteworthy that the chromosomes which reappeared were frequently distorted in shape.

Carbon particles injected into the center of an untreated nucleus remained at the site of injection presumably because they were caught in the central jelly matrix in which the chromosomes are embedded. However, an injection made just under the nuclear membrane of an untreated nucleus, where the nuclear matrix is relatively fluid, resulted in a scattering of the carbon particles between and around the parts of the chromosomes in the peripheral region. None of the particles penetrated the central region of the nucleus. Exposure of such an injected nucleus to an alkaline or hypotonic Ringer's solution or to distilled water, caused the chromosomes to disappear. The fact that the chromosomes still retained their identity and did not go into solution was shown by the alignment of the carbon particles which kept their relative positions outlining the contiguous borders of what must be the swollen chromosomes (Fig. 5). The carbon particles failed to become completely dispersed

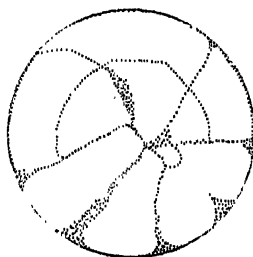


FIGURE 5. Alignment of carbon particles outlining the contiguous borders of adjacent chromosomes in an optically homogeneous nucleus. The nucleus was first injected with a carbon suspension, then hyalinized by immersion of the gland in hypotonic Ringer's solution.

as would be expected if the chromosomes had gone into solution. In nuclei which were first made hyaline, then injected with the carbon suspension in the peripheral region, a similar alignment of the carbon particles resulted. In both cases the chromosomes reappeared in the expected positions when the glands were placed in Ringer's solution.

It may be concluded, therefore, that although moving microneedles back and forth within the hyaline nucleus fails to reveal the presence of the swollen chromosomes, nevertheless the chromosomes have maintained their morphological integrity as shown by the injection of carbon particles.

Isolated chromosomes

The physical properties of the isolated chromosomes may be seriously affected by the methods used in their isolation and by the medium in which they are maintained. In the following experiments, attempts were made to maintain the properties which had previously been found for the chromosomes within the nucleus. It was found that rapid isolation of the chromosomes without contact with the torn cytoplasm was essential to prevent changes in structure and physical properties. Chromosomes which remained in the torn cell or came in contact with the cytoplasm during the isolation were shrunken, often distorted, and highly sticky. Of the various media tried, the one which caused the least change in the chromosomes after their isolation was the neutral KCl-NaCl solution described in the section on methods. In the experiments which follow on the isolated chromosomes, it is understood, unless otherwise stated, that the chromosomes were isolated into the neutral KCl-NaCl solution without coming in contact with the torn cytoplasm, transferred to a fresh drop of the same solution, and then manipulated. The duration of an experiment was not more than 10 minutes.

Stickiness. Chromosomes isolated into the neutral KCl-NaCl solution. Ringer's solution or hemolymph, were not unduly sticky. It was found that alkalinizing the medium (pH 8.0) caused the chromosomes to become sticky, whereas acidifying the medium (pH 6.5) reduced stickiness.

Consistency. The consistency of the isolated chromosomes in slightly alkaline KCl-NaCl solutions, pH 7.6-7.8, was found to resemble most closely that of the chromosomes in the intact cell, but structure was indistinct and the chromosomes were sticky. As the pH was decreased, the chromosomes shrank and the consistency increased. In neutral solutions little shrinkage occurred and the consistency was but slightly greater than that of the chromosomes within the intact cell. In acidic solutions (pH 6.0-6.5), the effects were more marked. At corresponding pH values, the consistency was always somewhat greater in Ringer's solution and in hemolymph than in the KCl-NaCl medium.

Chromosomes maintained in the neutral KCl-NaCl solutions were, like those in the intact nucleus, soft easily deformable gels which could be compressed without buckling. When they were cut or pulled apart by microneedles the cut or broken ends assumed smooth, rounded contours. This is in marked contrast to the results obtained with chromosomes which have been isolated after exposure of the glands to osmic acid vapors, formaldehyde, or acetic acid, following the methods of Buck (1942). Even brief treatment has a pronounced effect on the consistency of the chromosomes, causing them to become relatively rigid bodies which break squarely across when stretched.

It may be concluded that, although the consistency of isolated chromosomes maintained in a slightly alkaline KCl-NaCl solution most closely resembles that of the chromosomes within the cell, a neutral medium is preferable for manipulation since structure is still maintained and stickiness is lessened.

Extensibility and elasticity. The use of isolated chromosomes permits unrestricted stretching which obviously is impossible in the intact cell. It was found possible to stretch an isolated chromosome 10 times its original length and obtain complete recovery on release of tension. During the process of stretching, the interbands stretched somewhat more readily and so underwent a greater decrease in diameter than the bands. The wide bands separated into two or more narrow bands as occurred in the intact cell operations. As stretching was increased the diameter of the chromosome decreased so that at 10 fold elongation it appeared to be a mere thread with occasional nodular swellings. If this degree of stretching was not repeated more than 2 or 3 times, it was completely reversible. Repeated stretching caused permanent deformation, particularly of the interband regions, which remained in a partially stretched condition even after tension was released. Much greater stretching was possible without causing a chromosome to break in two. In some cases, a chromosome could not be broken within the limits of stretching of the micromanipulator. Thus one chromosome was stretched 25 fold without breaking. When tension was released, it returned to $1.3 \times$ its original length. Other chromosomes broke in two when stretched 12 fold. The break always occurred in an interband region, although this was not apparent until the chromosome had partially recovered. Fibrillae were occasionally seen at the broken ends. It should be pointed out that greatly stretched chromosomes developed a coarse fibrillar structure and rapidly deteriorated. This fibrillar appearance was different from that observed in unstretched chromosomes in that these fibrils were relatively coarse, and always followed the lines of tension. Deterioration also occurred, although much more slowly, in chromosomes which were not manipulated, indicating that the medium is not all that is to be desired.

It was found that on the addition of acids (pH 6.0–6.5) or calcium ions to the KCl–NaCl medium, the consistency of the bands was increased more than that of the interband regions. This effect was especially marked in the acid solutions, the bands becoming curved or bent rather than stretching or separating into narrow bands when placed under tension. The interband regions, although having a higher consistency than in neutral solutions, nevertheless were readily extensible, and when stretched more than twice their length appeared coarsely fibrous. At a 5–10 fold elongation, a chromosome broke in an interband region as before with fibrillae being evident at the broken ends. In slightly alkaline solutions, pH 8.0, the chromosomes became extremely ductile and showed almost no elasticity. The loss of elasticity was partially prevented by the addition of calcium salts to the alkaline solutions.

Isolated chromosomes were also stretched laterally. These gave results qualitatively similar to those observed in the intact cell. The isolated chromosomes, however, could not be stretched more than approximately double their width without deteriorating. In some instances, at least, the deterioration was associated with tearing the chromosome membrane.

In some of the experiments Buck's (1942) method was used of exposing glands to osmic acid vapors for 18–24 hours at 5° C., then isolating the chromosomes in distilled water. The chromosomes could be isolated readily by this method, and, in agreement with Buck, were tough, relatively rigid bodies, breaking squarely across in an interband region when stretched more than four times their length. Occasional fibrillae appeared extending from the broken ends of the chromosomes. This

was also observed by Buck, although he attached no particular significance to it. Stretching was reversible only up to 2 fold elongation (or 100 per cent as stated by Buck). Thus these chromosomes were far less extensible and elastic than those in the fresh state.

Structure. The chromosomes isolated in the KCl-NaCl solution at pH 7.0 generally showed no structure either in the interband regions or in the bands. Occasionally fibrillae were visible in the interband regions. On the other hand, in an acid solution (pH 6.5), fibrillar structure was more generally evident. In those cases where the fibrillar structure was already apparent, it became more distinct when the medium was slightly acidified.

Isolated chromosomes were dissected in the same manner as the chromosomes in the intact cell. The fibrils removed from the isolated chromosomes, behaved, except for a slightly increased stiffness, similarly to those removed from the chromosomes within the nucleus.

Occasionally it was found possible to produce a breakdown of the chromosomes into numerous longitudinal fibrils. This was done as follows: an intact gland was immersed in a hanging drop of neutral KCl-NaCl or in amphibian Ringer's solution, then a large pipette, about 15μ in diameter, was inserted into the nucleus of an intact cell and a single chromosome withdrawn. This could be done readily, indicating the ease with which the nuclear matrix becomes solated, and the relative non-adhesiveness of the chromosomes to each other. The chromosome was then expelled into the drop to one side of the gland, whereupon it sometimes became shredded into numerous longitudinal fibrils which resembled the fibrils obtained by dissection of an intact chromosome. This operation was successful only if the bore of the pipette was close to the diameter of the chromosome, thus suggesting the possibility that the chromosome membrane may have been removed while the chromosome was being either sucked into or expelled from the pipette. If the pipette was much larger than the chromosome, then the chromosome was expelled intact. The fibrils of the shredded chromosome were very numerous, sometimes appearing as a jumbled mass, and sometimes as a bundle of essentially parallel longitudinal fibrils. This experiment was repeated several times on the same day and on different days. The operation was not successful every time but the fault could be ascribed to the critical bore of the pipette and the speed with which ejection was made. The fraying of these chromosomes occurred irrespective of whether the medium into which they were expelled was NaCl-KCl or amphibian Ringer's solution. The operation was not done a sufficient number of times to discover an optimum medium for the fraying. The fact that it can be made to occur even very occasionally is highly significant.

DISCUSSION

The results of these micrurgical experiments indicate that the normal structure and physical properties of salivary gland chromosomes can be maintained only if due precaution is taken to avoid the deleterious effects of torn cytoplasm. The "acid of injury" reaction of torn cytoplasm, originally described by Chambers (1924b), is significant in this regard. More recently, Duryee (1941) has reported the injurious effects of torn cytoplasm and calcium ions on the isolated chromosomes and germinal vesicles of amphibian oocytes. The changes in structure and physical

properties of the salivary gland chromosomes which occur in a torn cell appear to be similar to those effected by acid or calcium ions. This suggests that calcium and hydrogen ions may be released from the injured cytoplasm.

The general finding that chromosomes isolated in media of a physiological pH are sticky raises the question as to why they do not adhere to each other within the normal nucleus. It has previously been shown (Glancy, 1940) that the chromosomes within the salivary gland nucleus are normally held apart by a thixotropic jelly matrix which becomes solated when the cell is torn. Although the chromosomes in the torn cell tend to become progressively sticky, this change is minimized if they are immediately removed from the cell so that contact with the torn cytoplasm is avoided. With prolonged contact, however, the chromosomes become so sticky that it is virtually impossible to manipulate them. The latter observations may indicate that the stickiness of isolated chromosomes is caused in part by chemical substances liberated from the disintegrating cytoplasm.

The reports of various investigators (Vonwiller and Audova, 1933; Barigozzi, 1938; Stefanelli, 1939; and Pfeiffer, 1940) to the effect that isolated chromosomes are tough, viscid gels, are in contrast to the observations in this paper which indicate them to be soft, easily deformable gels. The discrepancy may be explained in part by the failure of these investigators to observe precautions with respect to injured cytoplasm and the medium. My observations show that the consistency of isolated chromosomes tends to be greater than that of those within the nucleus, especially in the presence of injured cytoplasm. This increase in consistency seems to be associated with a simultaneous shrinkage of the chromosomes. Since the above investigators manipulated the chromosomes in the same drop of hemolymph or Ringer's solution in which the isolation had been performed, it is likely that the drop may have been acid because of the presence of the injured tissues. Even slight acidification of the medium has been shown to cause an increase in consistency. A similar effect results from the presence of calcium ions. Osmic acid vapors, used by Buck (1942) in treating the salivary glands prior to isolation of the chromosomes, cause far greater changes in physical properties although no shrinkage occurs. The findings of Buck, which we have confirmed using his technique, to the effect that the chromosomes are tough, cannot be compressed without buckling, and that they break squarely across when stretched, are at variance with those obtained on fresh chromosomes as shown in this investigation. It is thus evident that the consistency of the chromosomes is influenced by many factors.

The relative consistency of the bands and the interband regions has been discussed ever since Balbiani (1881) first suggested the bands to be gel and the interband regions, sol. Guareschi (1939) has observed that in dark field only the thicker bands are visible, whereas the thin bands and interband regions are not visible, from which he inferred that the former are in a gel state, and the latter in a weak gel, or more probably a sol state. Our microdissection experiments confirm Guareschi's inferences as to these differences in consistency. The thin bands and the interband regions must be weak gels rather than sols, however, for a puncture made in these regions leaves a temporary opening, a condition which does not obtain in a sol. This observation is further strengthened by the high elasticity of the chromosomes for, while gels are generally very elastic, a sol has negligible elasticity.

The extensibility and elasticity of the chromosomes have been studied by numerous investigators. Since it is well known that the properties of a colloidal structure

depend to a large extent on the medium, it is to be expected that the conditions under which the chromosomes are examined will influence their degree of elasticity. It is to be noted that the stretching experiments of Vonwiller and Audova (1933), Barigozzi (1938), Stefanelli (1939) and Pfeiffer (1940) were performed in 0.6 per cent NaCl, Ringer's solution, or hemolymph, without regard for the effects of the torn cytoplasm. These investigators found that the chromosomes could be stretched many times their length, though not reversibly. Pfeiffer and Stefanelli found stretching was not reversible beyond a 2 fold elongation as did Buck (1942) using osmicated chromosomes. It has been shown in this investigation that the elastic limit of fresh chromosomes in the intact cell is at least 5 fold, and when isolated in a KCl/NaCl medium, as much as 10 fold. This unusually high elasticity indicates the presence of long fibrous molecules (Mark, 1941) of which nucleoproteins are a characteristic type (Mirsky and Pollister, 1942).

The presence of a membrane on the salivary chromosomes has been described heretofore from the appearance of fixed and stained chromosomes (Schultz, 1941; Kodani, 1942). Metz (1934) believed the presence of a sheath or membrane to be necessary on theoretical grounds. Painter (1941) originally considered a pellicle to be present, but finally concluded, after studying the action of alkaline solutions on the salivary chromosomes, that the chromonemata are held together by a matrix, and that a pellicle is lacking. That the fresh chromosome possesses a membrane is strongly suggested by my microdissection experiments and observations that the outer boundary of the chromosome ruptures at a critical diameter when stretched by fluid injections. The membrane may aid in preventing contact between the chromosomes, since they tend to become sticky if it is torn, or it may serve to hold the chromonemata together.

The question as to whether or not the chromosomes in hyaline nuclei maintain their morphological integrity has frequently been raised. Stefanelli (1939) and Guareschi (1939), studying salivary gland nuclei rendered hyaline by immersion of the gland in hypotonic solutions, concluded that the chromosomes are completely dispersed. Stefanelli's conclusions, based primarily on the observations that microneedles can be moved back and forth within the hyaline nucleus without meeting resistance and that the nucleolus can be displaced without rebounding, do not conclusively demonstrate that the chromosomes are dispersed, however. The failure to find evidence for chromosomes by this technique might be explained in several ways: first, movement of the microneedles within the thixotropic nucleus might well induce solation; secondly, the consistency of the nuclear matrix in such nuclei may be close to that of the chromosomes; and thirdly, the swollen chromosomes are closely pressed against the nuclear membrane, leaving the center of the nucleus almost devoid of any resisting structure. To Guareschi's conclusions, it may be objected that a dilute gel cannot necessarily be distinguished from a sol under dark field illumination, and secondly, that the refractive indices of the colloidal particles in the chromosomes and the surrounding nuclear matrix may have been changed by the hydration which occurs in hypotonic solutions. Micro-injection results on salivary gland nuclei, likewise made hyaline by hypotonic solutions, indicate that the chromosomes, though invisible, maintain their morphological integrity, since injected carbon particles clearly indicated the outlines of the chromosome cylinders. If the chromosomes were completely dispersed, the injected carbon particles should be distributed at random in the nucleus.

The polytene concept of chromosome structure rather than the alveolar is supported by the various observations made in this investigation. The controversy regarding the structure of the salivary chromosomes results in part from the fact that fixed and stained chromosomes do not always present a similar appearance with regard to the finer details. Since the striations are especially evident in stretched chromosomes, Metz (1941) believes them to be artifacts produced by drawing out the walls of alveoli. Buck (1942) has provided experimental support for the alveolar hypothesis. It is agreed that a fibrillar-like appearance in homogeneous-appearing chromosomes can be produced by stretching. However, I have never observed a pre-existing alveolar structure in fresh chromosomes, nor does Buck state that the alveolar condition was present before he treated the glands with osmic acid vapors, although he does state that the treated chromosomes closely resemble those in the living animal. Most of his photomicrographs fail to show an alveolar structure. It is conceivable that stretching the chromosomes could cause aggregation of delicate, scarcely visible or non-visible fibrils, into the relatively coarse fibrils which are commonly seen in stretched chromosomes. Buck tried unsuccessfully to shred the chromosomes into fibrillae. This may easily be accounted for by the fact that he used osmicated chromosomes which are of such high consistency that shredding is impossible.

A fibrillar appearance was occasionally noted in the fresh salivary chromosomes studied in this investigation. Buck (1942) also reported that fresh chromosomes may occasionally show longitudinal striations, but he attached no particular significance to this observation. In chromosomes which show no structural details in the interband regions, a slight shrinkage produced by mild acidification (pH 6.5) results in the appearance of delicate longitudinal striations but never alveoli. Since the chromosomes have not been stretched, it does not seem likely that the fibrillar appearance can be explained on a basis of drawn out alveoli. The observation that fibrillae may appear at the broken ends of greatly stretched chromosomes may be interpreted in two ways, as Buck (1942) pointed out when he made a similar observation on osmicated chromosomes. The fibrillae may have been pre-existing, or they may be artifacts produced by the stretching. More significant is the microdissection from fresh unstretched chromosomes, either within the intact nucleus or after isolation, of delicate longitudinal fibrils which give the appearance of chromonemata. It is difficult to see how such fibrils could be removed from an alveolar mesh. Attempts to remove transverse fibrils, which should be equally possible if an alveolar structure exists, were uniformly unsuccessful. The finding that a fresh chromosome, expelled from a micropipette into Ringer's or KCl-NaCl solution, may break up into numerous delicate longitudinal fibrils, but never an alveolar mesh, is also difficult to explain except on a polytene basis.

SUMMARY AND CONCLUSIONS

Micurgical experiments performed on the fresh salivary gland chromosomes of mature *Chironomus* larvae within the nucleus of the intact cells and after their isolation into a specially designed medium indicate the following:

1. Puncture of the cell with a fine microneedle has little or no effect on the structure and physical properties of the chromosomes provided tearing of the cytoplasm is prevented.

2. Tearing of the cytoplasm causes the chromosomes to shrink markedly and become highly viscid.

3. The structure and physical properties of chromosomes isolated into a medium consisting of 0.09 M KCl, 0.06 M NaCl, and 0.005 M phosphate buffer at pH 7.0, are not appreciably altered, except for a slight increase in consistency, from those of chromosomes in the intact cell if precaution is taken to prevent contact of the chromosomes with the torn cytoplasm.

Physical properties

4. Evidence for stickiness of the chromosomes can be obtained only when the thixotropic jelly matrix surrounding them is isolated.

5. The chromosomes are soft, easily deformable gels with interband regions of lower consistency than the bands.

6. The chromosomes are highly extensible and elastic. They can be stretched 5 fold within the intact cell without permanent deformation. Greater stretching cannot be accomplished without tearing the nuclear membrane. Isolated chromosomes regain their initial length after 10-fold elongation, but excessive stretching ($12-25\times$) causes the chromosomes to break in an interband region.

7. Modifications in the properties of the chromosomes are easily induced by chemical agents. Dilute alkalis decrease consistency and elasticity, but increase extensibility. Dilute acids and calcium ions increase consistency, and decrease extensibility and elasticity. Osmic acid and formalin have a similar but much greater effect.

8. Chromosomes in nuclei hyalinized by hypotonic or alkaline Ringer's solution retain their morphological integrity.

Structure

9. The fresh chromosome possesses a delicate elastic membrane.

10. Chromosomes, either in the intact nucleus of the unoperated cell, or when isolated, occasionally display delicate longitudinal striations in the interband regions, but never show alveoli.

11. Slight acidification of the medium to pH 6.5 causes the appearance of similar striations in the interband regions of unstretched chromosomes.

12. Chromosomes broken in two by stretching frequently exhibit fibrillae at the broken ends.

13. Delicate fibrils may be dissected from the intact or isolated chromosome. The fibrils possess nodular swellings at intervals corresponding to the bands.

14. A chromosome drawn up into a micropipette of optimal size, then expelled into Ringer's or KCl-NaCl solution, may shred into numerous fibrillae.

These observations are best interpreted as supporting a polytene concept of chromosome structure.

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The amount of fluid used varied with the size of the animals. Protozoa and certain other forms were tested in watch glasses containing 10 ml. of fluid. Other forms were tested in shell vials or finger bowls containing 25–200 ml. of fluid. Of course, single series and replicates were with uniform volumes. Continuously flowing suspensions were not feasible; the more difficultly cultured marine animals were changed to fresh suspensions twice daily. For species that did not require frequent changing of the medium, subsequent assays with mosquito larvae or hermit crabs were commonly employed to verify the continued toxicity of test fluids.

Specimens were obtained from various sources. Protozoa were from laboratory cultures and were identified by Dr. D. H. Wenrich. Marine forms were collected at Woods Hole, mostly by the Supply Department, and the names used are those current at that laboratory. Other forms were collected or purchased, and identified by the authors.

Chemical tests for the presence of chitin were routinely performed on exoskeletons using the methods given by Campbell (1929). Positive results were obtained with Arthropods and the perisarc of Colenterates. The Bryozoan used (*Bugula*) gave atypical results suggesting a skeleton composed of something similar to, but not, chitin.⁵

For adsorption tests cuticle was removed from Horseshoe Crabs (*Limulus polyphemus*), manually cleaned of cells, rinsed thoroughly with distilled water, and dried. Part of this material was treated with 5 per cent NaOH at 100° C. until the color was removed and the pieces were negative to protein tests. This latter material is referred to herein as "chitin" although tests showed that a small percentage of the material had been changed to chitosan (see Campbell, 1929). Smaller amounts of cuticle were prepared from cockroaches and used in a few experiments, but most of the adsorption work was done with the more available cuticle of *Limulus*.

All tests were performed in replicate. Many of them were repeated.

RESULTS

The results obtained are summarized in the accompanying table. In all cases the toxin (DDT) was applied as a colloidal suspension in the bathing medium. The resistances or susceptibilities shown refer only to the indicated concentration in the surrounding medium. The figures are not comparable to median lethal doses and should not be interpreted as implying that "resistant" animals would be resistant to the injection of solutions or emulsions.

⁵ The cuticle of *Bugula* is variously referred to in the literature as "chitinous" or "material akin to chitin." We applied the various chemical tests used by Campbell (1929) and others. The cuticle is not dissolved in hot concentrated KOH. After prolonged heating (which changed control pieces of known chitin to chitosan completely) it only crinkled in 3 per cent acetic acid instead of dissolving. The addition of one per cent H₂SO₄ gave no change and no precipitate. Another piece treated with I + KI in water turned brown but only very slowly. After the addition of one per cent H₂SO₄ it remained brown for several minutes and then after 5–10 minutes slowly changed to a *greenish-brown* (instead of the clear violet given by chitosan). It dissolved immediately in 75 per cent H₂SO₄, but after standing no crystals resembling chitosan sulfate crystals were found. These tests suggest a similarity to chitin but certainly, at least in this species, the bryozoan cuticle is not true chitin.

TABLE I
The effective toxic concentrations of DDT for various animal phyla

All tests were replicated at least once. Susceptible species were tested repeatedly. Except where indicated otherwise tests were continued for 7 days or longer. The fluid on species that do not survive well in stagnant water was renewed daily or more frequently.

Group	Species	Temperature, C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Protozoa	<i>Ameba proteus</i>	room	fresh	—	—	—	No effect (14 da.)	Slow effect?	Slowly killed (3-10 da.)
	<i>Paramecium</i> sp.	room	fresh	—	—	No effect	No effect	No effect (1 mo.)	No effect (1 mo.)
	<i>Spirogyra</i>	room	fresh	—	—	—	—	—	—
	<i>Euglena gracilis</i>	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
	<i>Chlamydomonas</i> sp.	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
Coelenterata	<i>Peranema</i> sp.	room	fresh	—	—	—	No effect	—	No effect (1 mo.)
	<i>Obelia</i> sp. ¹	15°	sea	—	Many killed	Killed (1-2 da.)	Killed (<1 da.)	—	—
	<i>Campanularia</i> sp. ¹	15°	sea	—	No effect	Affected slightly ²	Affected ³	—	—
	<i>Tubularia</i> sp.	15°	sea	—	No effect	Killed slowly	Killed	Killed	—
	<i>Penaria</i> sp.	15°	sea	—	No effect	No effect	No effect	Affected ³	—
Platyhelminthes	<i>Hydractinia</i> sp.	15°	sea	—	No effect	No effect	No effect	Affected ³	—
	<i>Hydra fusca</i>	15°	fresh	—	No effect	No effect	No effect	Affected ³	—
	<i>Astrangia</i> sp.	15°	sea	—	—	No effect	No effect	—	—
	<i>Phragmatia</i> sp.	room	fresh	—	—	No effect (1 mo.)	Killed (7-40 da.)	Killed (7-20 da.)	Killed (1-6 da.)
	<i>Anguillula aceti</i>	room	fresh	—	No effect	No effect	No effect	No effect	No effect (1 mo.)
Nematoda	<i>Ascaris lumbricoides</i>	35°	alkaline saline	—	No effect	—	No effect	No effect	No effect
	<i>Phidippa</i> sp.	room	fresh	—	No effect	No effect	No effect	No effect (1 mo.)	No effect
Rotifera	<i>Typhlos</i> sp.	room	fresh	—	—	—	Some killed slowly (1-2 mo.)	No effect (1 mo.)	No effect
	<i>Monostyla</i> sp.	room	fresh	—	—	—	—	Killed slowly (10-30 da.)	Killed slowly (7-10 da.)
Gastrotricha	<i>Chaetonotus</i> sp. ⁴	room	fresh	—	—	No effect	No effect	—	—
	<i>Bugula</i> sp.	15°	sea	—	No effect (10 da.)	Killed slowly (4-6 da.)	Killed slowly (3-4 da.)	—	—

¹ At least two, probably three, species of *Obelia* and *Campanularia* were used in these tests but identification of species in this group is quite difficult.

² *Tubularia* stems selected by Dr. L. G. Barth were set-up as in regeneration studies. All specimens regenerated but polyps quickly died in stronger concentrations and almost without exception did not regenerate a second time. Controls regenerated repeatedly.

³ At 1:100,000 the specimens of *Hydra*, *Hydractinia*, and *Astrangia* were rounded and slow in reacting at the end of six days but were still living.

⁴ These gastrotrichs were not tested separately. They were present in one of the rotifer cultures, and data cover only those test dishes, in which they were found.

TABLE 1—Continued

Group	Species	Temp. air, °C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Mollusca	<i>Littorina</i> sp.	room	sea	—	No effect	No effect	No effect	No effect	Killed slowly (6-7 da.)
	<i>Mytilus</i> sp. ⁶	15°	fresh	—	—	—	—	—	No effect
	<i>Patersonia trilineata</i>	25°	fresh	—	—	—	—	—	No effect
	<i>Physa</i> sp.	25°	fresh	—	—	—	—	—	Killed slowly
Echinodermata	<i>Thyone</i> sp.	room	sea	—	—	—	No effect	No effect	No effect
	<i>Asterias</i> sp.	room	sea	—	—	—	No effect	Killed (2 da.)	No effect
Annelida	<i>Aeolosoma</i> sp. ⁷	room	fresh	—	—	—	No effect?	Effect? *	—
	<i>Polydora</i> sp. ⁸	15°	sea	—	—	—	No effect?	No effect (7 da.)	Killed (2-3 da.)
	<i>Nereis virens</i>	15°	sea	—	—	—	No effect	Killed (4 da.)	No effect
	<i>Lumbricus terrestris</i>	15°	fresh	—	No effect	—	—	No effect	No effect
Crustacea	<i>Artemia salina</i>	15°	sea	Most killed (2-6 da.)	Killed (2-6 da.)	Killed (1-5 da.)	Killed (1-5 da.)	Killed (2-5 da.)	Killed (1-5 da.)
	<i>Gammarus</i> sp.	15°	fresh	Most killed (1-6 da.)	Killed (1-3 da.)	Killed (1-3 da.)	—	—	—
		28°	fresh	<half-killed (6 da.)	Killed (1-3 da.)	Killed (1-2 da.)	Killed (1 da.)	Killed (<1 da.)	—
	<i>Daphnia</i> sp.	15°	fresh	Most killed (2-7 da.)	Killed (1-5 da.)	Killed (1-5 da.)	—	—	—
	<i>Cyclops</i> sp.	room	fresh	No effect	No effect?	No effect?	Killed (<3 da.)	Killed (<3 da.)	Killed (<3 da.)
	<i>Pagurus</i> sp.	15°	sea	—	—	—	Killed (1-2 da.)	Killed (1-2 da.)	—
	<i>Emerita talpoida</i>	15°	sea	—	—	—	—	—	—
	<i>Aedes aegypti</i> larvae	15°	fresh	Half killed (7 da.)	Killed (2-7 da.)	Killed (2-4 da.)	Killed (2-4 da.)	—	—
Insecta		28°	fresh	No effect (7 da.)	Most killed (7 da.)	Killed (1-4 da.)	Killed (1-2 da.)	—	—
	<i>Culex pipiens</i> larvae	15°	fresh	—	—	Killed (<1 da.)	—	—	—
		30°	fresh	—	—	Killed (1-3 da.)	—	—	—
	<i>Chironomus</i> sp. larvae	room	fresh	—	—	Killed (1-2 da.)	—	—	—
	<i>Chironomus</i> sp. larvae	10°	fresh	Some killed (5 da.)	Killed (5 da.)	Killed	—	—	—
		30°	fresh	No effect (5 da.)	Most killed (5 da.)	Killed	—	—	—
Fungi	<i>Rhizopus nigricans</i>	room	fresh	—	—	—	—	—	No effect
	<i>Penicillium</i> sp.	room	fresh	—	—	—	No effect	No effect	—
	<i>Saccharomyces cerevisiae</i>	room	fresh	—	—	—	—	—	—

* Only a few specimens (not a statistically significant number) were tested of *Mytilus* and *Polydora*. The negative results suggest that these are both resistant species, as are other members of their phyla.

⁶ At 1:100,000 there was an initial decrease in numbers followed by an increase. It would seem that some are killed but that the survivors reproduce readily.

⁷ The earthworms were tested in flat dishes with the fluid not sufficiently deep to cover the animals completely.

⁸ Anderson (1945) records an effect on *Daphnia* at concentrations greater than one part per billion and no effect at concentrations lower than one part per billion. Apparently his cultures were slightly more susceptible to DDT than ours.

Of the species tested, the Protozoa, Nematoda, Gastrotricha, Rotifera, Mollusca, Annelida, and Echinodermata were resistant to DDT suspensions or in the case of a few species, were slowly killed by very concentrated suspensions. The single species of the Platyhelminthes tested was comparatively resistant. The single species of Bryozoa was quite susceptible. And, the various species of Arthropoda and those Coelenterata with a complete perisarc were highly susceptible. The most interesting data come from the hydrozoan Coelenterata where those species with a complete chitinous perisarc (*Obelia* and *Campanularia*) are highly susceptible, those species with a chitinous perisarc over only the main stalk (*Tubularia* and *Pennaria*) were less susceptible, and those without a perisarc (*Hydra* and *Hydractinia*) were nearly resistant. The bryozoan, *Bugula*, is an intermediate which is almost as susceptible to DDT as the arthropods and sensitive coelenterates. *Bugula* has a complete cuticle which is composed of some substance seemingly similar to but not identical with chitin.⁵ Correlated with this similarity is the more or less similar relationship to DDT poisoning from dilute solutions. The most reasonable assumption at present is that the cuticle of *Bugula* behaves towards DDT similarly to chitinous cuticles but that it is less effective (or possibly the animals are really more resistant).

The data presented herein do not include vertebrates. The most nearly comparable data on fishes indicate an intermediate sensitivity (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945) and that concentrations entirely adequate for mosquito control (i.e., well above the minimum lethal concentration) do not injure fish (Metcalf *et al.*, 1945). With terrestrial vertebrates truly comparable data are not available. The closest parallel is with aerosols and mists; very high concentrations of DDT applied in this manner cause little or no injury to mammals (Neal *et al.*, 1944). DDT in solution in oil can be absorbed through mammalian skin (Draize *et al.*, 1944) but the dosage needed to produce an effect is fairly high. Also, it is well known that DDT has been approved for use as a powder on vertebrates to kill ectoparasitic insects. The data, then, suggest that for external applications more or less comparable to our data, fish occupy an intermediate position in susceptibility—being some ten to one hundred times more resistant than mosquito larvae. Mammals seem to be much more resistant to external applications than either fish or insects.

The obvious correlation of the above data is to the chitinous exoskeleton. Actually there are a number of possibilities, notably (1) that some of the animal groups are truly more or even completely resistant to the toxin; (2) that certain animal groups may be able to detoxify or excrete DDT better than others; (3) that there may be a reaction between a chitinous cuticle and DDT to produce some other more toxic substance; or (4) that the susceptibility may be due solely to penetration and accumulation which is favored by a chitinous cuticle. The evidence at hand suggests that both the first and last named possibilities are partly correct. Considering the points in order:

Data derived from the feeding and injection of DDT solutions (not suspensions) and emulsions show that on a dosage/weight basis mammals have approximately the same order of susceptibility as cockroaches (Draize, *et al.*, 1944; Smith and Stohlman, 1945; Chadwick, 1945). While some variability is obtained among insects (Chadwick, 1945), the fact remains that internal applications do not

show the tremendous differences between vertebrates and insects that external applications show.

However, there must be a considerable amount of variability in true resistance among different groups on invertebrates. *Ascaris* is immune to external applications of DDT suspensions but the median lethal dose for injection of emulsions (on a dosage/weight basis) seems at least several times as high as the MLD for cockroaches.⁶ And, injection of DDT emulsions into the common aquarium snail, *Helisoma trivolva*, was apparently without effect even when one mg. of DDT was injected into a snail whose weight (without the shell) was less than a gram. Unfortunately, we have no precise knowledge of the site of injection but at least it was into the interior of the animal and sometimes into the tissues. It seems clear from these data (1) that there must be considerable difference among various animals as to the effect of a given amount of DDT after it is *in* the animal, and (2) that relative absorbability also varies.

The second possibility, detoxification or excretion by certain groups, seems unlikely both because of the similarity of vertebrate and insectan median lethal doses on injection and because of the great dissimilarity in excretory organs. The role of detoxification may actually be considerable. One non-toxic breakdown product, dichlorodiphenylacetic acid, has already been positively identified in mammalian urine (White and Sweeney, 1945). Detoxification, however, does not seem to contain the full answer to the wide variations in susceptibility reported in this paper.

The third possibility, a new and more potent toxin arising from a reaction with chitinous cuticles, seems eliminated by several points. The above mentioned similarity of median lethal doses and symptoms following injection of emulsions is against it. Also pertinent is the fact that DDT suspensions have no visible effect on cultured tissues whether chitin particles are added to the medium or not (Lewis and Richards, 1945).

The above considerations led to two different types of tests on the fourth possibility; namely, that cuticles might facilitate the entry of DDT into animals. If chitinous cuticles facilitate the entry of DDT it might be possible to find aquatic invertebrates which are immune to external applications but killed by injections. We have data on one such case. *Aparis* does not have a chitinous cuticle and is completely resistant to immersion in the strongest DDT suspensions. It is killed by the injection of emulsions although requiring a somewhat higher dose than cockroaches and vertebrates.

While these data on *Ascaris* are suggestive, they, of course, do not prove anything about chitinous cuticles. More direct data were sought by trying to find a possible mechanism by means of which chitinous cuticles might facilitate the entry of DDT into an arthropod or coelenterate. The following data suggest that such a mechanism does, indeed, exist and that adsorption by chitin plays an important role in it.

Obviously, DDT can penetrate insect cuticles since external applications can kill even when the possibility of ingestion is eliminated. Tests showed that DDT could be adsorbed from water by activated charcoal. DDT suspensions in water

⁶ We determined the median lethal dose for *Ascaris* only to a rough order of magnitude and so prefer not to cite a definite quantitative figure.

lost all toxicity to mosquito larvae after being stirred with charcoal and filtered. It follows from this that the DDT particles have been removed by the charcoal. Similarly, we found that DDT could be adsorbed from aqueous suspensions by arthropod cuticle and even better by purified chitin. The results are only qualitative; accordingly, tabular data are not presented. Cuticle and purified chitin from *Limulus* and from cockroaches were prepared as described in the section on methods. An excess of either material was added to a moderately dilute suspension of DDT in distilled water, equilibrated at 6° C., and filtered. The filtrate had its expected toxicity to mosquito larvae greatly reduced or abolished. From this bio-assay it follows that the cuticle or chitin had removed part or all of the DDT. Cuticle or chitin was then added to a more concentrated suspension, equilibrated at 6° C., filtered, and the pieces of cuticle or chitin were soaked twice in cold distilled water and filtered to wash off any DDT that might be present in the water on the surface or within the matrix of the pieces. The lots of cuticle and chitin were then soaked in hot distilled water (90° C.) adjusted to pH 3.8 with 0.1 molar H_2SO_4 , filtered, and the filtrate tested by the usual bio-assay method with mosquito larvae. Symptoms and mortality showed that DDT had been recovered. Since DDT could be removed and recovered by these adsorption methods it follows that cuticle and purified chitin are capable of adsorbing DDT.

In addition to the controls accompanying the above adsorption tests, a number of other things were tested. As judged by subsequent bio-assay a small amount of DDT was adsorbed by cleaned dry spines of sea urchins, but none was adsorbed by snail shells, coral, a suspension of erythrocytes (cow), or chunks of muscle (*Limulus*).

Another point which is consistent with the idea that adsorption by chitin facilitates the entry of DDT, is that mosquito eggs are resistant to any concentration of DDT tested, whereas, the larvae on hatching are susceptible. This might represent differences in embryonic stages or a barrier between the egg shell and the enclosed embryo or larva but it could conceivably be due to the fact that larvae have a chitinous cuticle, whereas, the insect egg shell contains no chitin.

If adsorption phenomena are playing an important role, one would expect to find a negative temperature coefficient, i.e., that DDT is more toxic at lower temperatures than at higher temperatures. Solubility tests show that DDT has a positive temperature coefficient for solubility in both polar and apolar solvents. Accordingly, a negative temperature coefficient is good evidence in support of an adsorption hypothesis. Actually the temperature relationships are complex and not fully understood. Data obtained to date show that at low concentrations a negative temperature coefficient is, indeed, obtained but that at higher concentrations this changes to a positive temperature coefficient. However, it seems logical that a concentrating mechanism would be most dominant at lower concentrations. Accordingly, the temperature data support the hypothesis that adsorption of DDT by chitinous cuticles acts as a concentrating mechanism. The shift to a positive temperature coefficient at higher concentrations would seem to imply that other factors are involved and that the adsorption by chitinous cuticles is only for concentrating the toxin, the actual toxic action of DDT being something else.

Most of the temperature studies have been performed using mosquito larvae at 15° and 28° C., but similar results have been obtained with the fresh water shrimp

Gammarus.⁷ Lindquist *et al.*, (1945) have independently found a negative temperature coefficient in spray work with adult flies, and during the past year it has come to be generally considered that DDT works better in insect control operations in cool climates than in hot climates. Attempts to obtain data with coelenterates at high and low temperatures failed because we were not successful in keeping the species available at Woods Hole alive in warm, non-running water.

DISCUSSION

From the data presented here, we conclude that the sensitivity of certain animal groups to DDT applied externally as a colloidal suspension in the bathing medium is correlated with the presence of a chitinous or similar cuticle in addition to true differences in tissue susceptibility. This correlation is supported by the similarity of toxicity to certain animals when emulsions are injected in contrast to dissimilarity from external applications, and by direct demonstration of adsorption of DDT by chitin and chitinous cuticles, and non-adsorption by certain other types of exoskeletons and tissues. The idea that adsorption processes are important is further supported by the demonstration of a negative temperature coefficient for toxicity at lower concentrations. The data seem to concern only the differences in sensitivity and not to explain the toxic action itself. The exceptions recorded herein (e.g., snails) show that the action of DDT is more complex but they do not invalidate the cuticle relationship. Accordingly, we propose as a hypothesis that chitinous cuticles act to selectively concentrate DDT from the surrounding medium by adsorption processes.

This hypothesis is in some respects similar to the old idea of penetration due to partition coefficients. Several authors have invoked the idea of partition coefficients to explain insecticide penetration (Wigglesworth, 1941; Richards and Weygandt, 1945). The present hypothesis for concentrating DDT varies in substituting an active adsorbing interface of chitinous cuticle. On theoretical grounds Hurst (1943) has suggested that the insect cuticle may adsorb toxins, as work by Castle (1936) had already indicated. The data in the present paper, however, give the first direct demonstration that a chitinous cuticle can adsorb an insecticide and that the adsorption may play a role in selective toxicity to different groups of animals.

Since one is inclined to think of the arthropodan cuticle as a solid sheet of material, it is natural to ask where sufficient surface is available for the demonstrated adsorption. A possible clue is given by Castle's (1936) work on the "oriented imbedding" of organic substances in chitin. This is a kind of adsorption process that apparently utilizes the surfaces of known intermicellar spaces. Perhaps the same intermicellar surfaces may be available for the adsorption of DDT.

Obviously, adsorption by the cuticle can be only part of the story of the action of DDT. Some mechanism is needed to transport the DDT from the cuticle to its effective locus inside the animal. We have no direct evidence as to what this mechanism is. Perhaps one can interpret the data from distribution of the radio-

⁷ More recently Dr. Hsing Yun Fan has made an intensive study of the negative temperature coefficient using both mosquito larvae and midge larvae (*Chaoborus* sp.). His data will be published subsequently.

active bromine analog (Hansen, Hansen, and Craig, 1944) to mean that DDT is more soluble inside insects than in water. Such a difference in solubility in connection with an actively adsorbing cuticle could account for a selective accumulation in insects and other groups with similar cuticles.

A careful consideration of reported exceptions is necessary. Many papers have been published on the use of DDT as an insecticide.² These papers, while showing a general susceptibility of insects to DDT, are mostly not comparable to the present paper. They are not primarily concerned with the resistance or susceptibility of the various species but with the practicability of controlling pest insects under natural conditions. A susceptible insect that is not reached by the insecticide in the field will not be killed. Also the thickness, dryness, nature of the epicuticle (external layer outside of chitinous cuticle), and other properties of the cuticle vary greatly from one group to another within the arthropods (Richards, 1944). One should expect variation in susceptibility with these variables. In some cases it seems clear that forms which are not satisfactorily controlled are nevertheless susceptible. For instance ticks and mites are said not to be well controlled by DDT (Cox, 1945) yet they are by no means resistant (e.g., Gouck and Smith, 1944; Smith and Gouck, 1944; Vargas and Iris, 1944, etc.), and at least one species (*Rhipicephalus sanguineus*) is readily controlled by DDT. In some other cases, notably certain beetles with thick, hard, dry cuticles, insects seem little or not at all affected. Since these are terrestrial species, the difficulty in obtaining data comparable to data presented in this paper is considerable. In the Crustacea there is a considerable range of variability; most species tested were highly susceptible but the copepods are much less susceptible (see also Anderson, 1945; Seagren, Smith, and Young, 1945). It seems certain that more and less resistant species do occur among arthropods even though available data refer principally to practical field tests. While it is possible to offer hypothetical explanations of these variations on the basis of differences in cuticle structure, variations in degree of effect seem to be a factor also (Chadwick, 1945). However, the known susceptibility of arthropods in general is so broad that we feel inclined to suggest that the variations will be explained without vitiating the hypothesis advanced in the present paper.

The fungi with chitinous cell walls (*Rhizopus*, etc.) are a true exception. They are apparently indifferent to DDT in the medium bathing them. This resistance cannot be evaluated until we are more certain as to the method by which DDT kills. One obvious possibility lies in the differences between plants and animals, particularly, the absence of a nervous system in plants.

Another line of exceptions concern the intermediate susceptibility of fishes (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945, and others). No explanation of this is offered, but there is no reason to assume that intermediation of a chitinous cuticle is the only means by which DDT can enter an aquatic animal.

The most serious exceptions, however, are those invertebrates which are not affected by the injection of emulsions (snails). These are species representative of a group unaffected by external applications. No explanation of this exceptional lack of effect on injection is offered in the present paper, but obviously the snails demonstrate beyond question that more is involved in the whole story of DDT action than just the facilitation of entrance by an appropriate cuticle.

SUMMARY

1. Throughout the animal phyla there is a correlation between the presence of a chitinous cuticle and susceptibility to external applications of DDT. Those aquatic animals with a chitinous cuticle (arthropods and certain coelenterates) are highly sensitive to external applications of DDT, other animals are not so susceptible although there is considerable variability.

2. The correlation to a chitinous cuticle is supported by studies on various coelenterates, on adsorption, and on temperature coefficients. Coelenterata with respectively a complete, partial, and no chitinous perisarc are respectively highly sensitive, somewhat sensitive, and nearly insensitive to DDT. DDT can be adsorbed by chitin and chitinous cuticles and at low concentrations shows a negative temperature coefficient for toxicity to arthropods.

3. From these data a hypothesis is proposed that chitinous cuticles facilitate the entry of DDT into the animal body by selectively concentrating the compound by adsorption phenomena.

4. This is the first demonstration that an insecticide can be adsorbed by chitinous cuticles and the first direct evidence that such adsorption actually can play a role in insecticide action.

5. The present paper does not consider the nature of the toxicity of DDT to protoplasm. The data given here refer only to the selective action of DDT as a function of penetration facilitated by chitinous exoskeletons. The shift to a positive temperature coefficient at higher concentrations, the lack of effect from injecting DDT emulsions into snails, and the variability in median lethal doses for injected DDT emulsions in different animals, all indicate that the selective adsorption of DDT by chitinous cuticles is only a part of the story of the toxic action of this compound.

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PROTOPLASMIC VISCOSITY CHANGES IN DIFFERENT REGIONS OF THE GRASSHOPPER NEUROBLAST DURING MITOSIS

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Protoplasmic viscosity has been studied by a variety of methods. Each has certain advantages and certain limitations, with regard to the kind of living material to which it is best suited and the accuracy of the results that it will give. These methods and their uses have been reviewed critically by Heilbrunn (1928, 1943) and therefore will not be considered in detail here. Of the several that have been employed the brownian movement method is probably best suited to viscosity studies involving limited regions of the single cell. It may be applied in either of two ways: 1) to calculate the absolute viscosity by measuring the displacement in one direction of the molecularly-bombarded particle and the time required to bring this about, certain other characteristics of the cell and its environment being known, or 2) to compare the viscosities of different regions of a cell at different mitotic stages or under different experimental conditions through observations of the relative speeds of brownian movement. The latter has been used in the present investigation.

The neuroblasts of the grasshopper embryo possess several advantages in such a study. Since they are situated on the ventral side of the embryo, they can be brought next to the cover glass in hanging-drop preparations and microscopic observations of the mitotically active, living cells can extend over several hours. All the cell features that are visible in the usual fixed and stained preparation can be seen, and many phases of the mitotic cycle can be identified readily. The cell is relatively large, measuring about 30 μ in diameter. It maintains a visible polarity from one mitotic division to the next, so that a given region can be located in any cell at any stage of division. The cytosome contains large numbers of mitochondria, which not infrequently find their way into the spindle during mitosis. By observing the brownian movement of these tiny bodies it is possible to compare the viscosities of the surrounding protoplasm of all non-nuclear parts of the cell at all stages of the mitotic cycle.

MATERIAL AND METHODS

Embryos of *Chortophaga viridifasciata* (De Geer) were prepared by the hanging-drop method previously described for this material (Carlson and Hollaender,

¹ The preliminary observations on which this study is based were made as Rockefeller Fellow in the Natural Sciences at the Genetics Laboratory of the University of Missouri in the winter of 1940-41 and at the Biological Laboratory, Cold Spring Harbor, in the summer of 1941. The work has been completed with the aid of a 1944-45 grant from the University Research Committee of the University of Alabama.

1944). The culture medium, which must be isotonic with the embryonic cells, is made up as follows:

NaCl	0.70 gm.
KCl	0.02 gm.
CaCl ₂	0.02 gm.
MgCl ₂	0.01 gm.
NaH ₂ PO ₄	0.02 gm.
NaHCO ₃	0.005 gm.
Glucose	0.80 gm.
H ₂ O (pyrex redistilled)	100.0 cc.

The pH of this solution is approximately 6.5, which is about the pH of the grasshopper egg yolk. A small amount of yolk is added to each hanging-drop to provide nitrogenous food materials for the cells.

Preparations were studied in a constant temperature box enclosing all of the microscope except the upper part of the body tube and arm. All observations were made at $26 \pm 0.5^\circ \text{C}$. The light used as a source of microscope illumination was passed through copper sulfate solution to filter out the heat.

Observations were made exclusively on neuroblasts. These cells are shown in representative mitotic stages in Figure 1, which is based on camera lucida sketches of living cells. It will be noted that the neuroblast deviates from the typical cell in two main respects. First, the nucleus is not spherical but roughly hemispherical with a central cytoplasmic core connecting the polar and apolar regions of the cell (Figs. 1A and 1B). Second, the cell divides unequally to form a small daughter ganglion cell and a large daughter neuroblast (Fig. 1J.) The great advantage of these cells is their large size, which makes it possible to observe and study in the living, unstained cell many structural features that are very difficult or even impossible to deal with in smaller cells.

The mitochondria of the neuroblast are spheroidal in shape and measure approximately $0.3\text{--}0.5 \mu$ in diameter.

TERMINOLOGY

Relation of viscosity to brownian movement.

The relation between the rate of brownian movement and viscosity is given by Einstein's equation

$$D_x^2 = \frac{RTt}{N3\pi\eta a}$$

in which D_x is the displacement of the particle along the x axis; R , the gas constant; T , the absolute temperature; t , the time; N , the Avogadro number; a , the radius of the particle; and η the viscosity expressed in poises. Accordingly, the viscosity of the dispersion medium varies inversely as the square of the displacement of a given particle along one axis.

The rapidity of brownian movement of the mitochondria in selected regions of the cell was observed and classified according to the following system. The

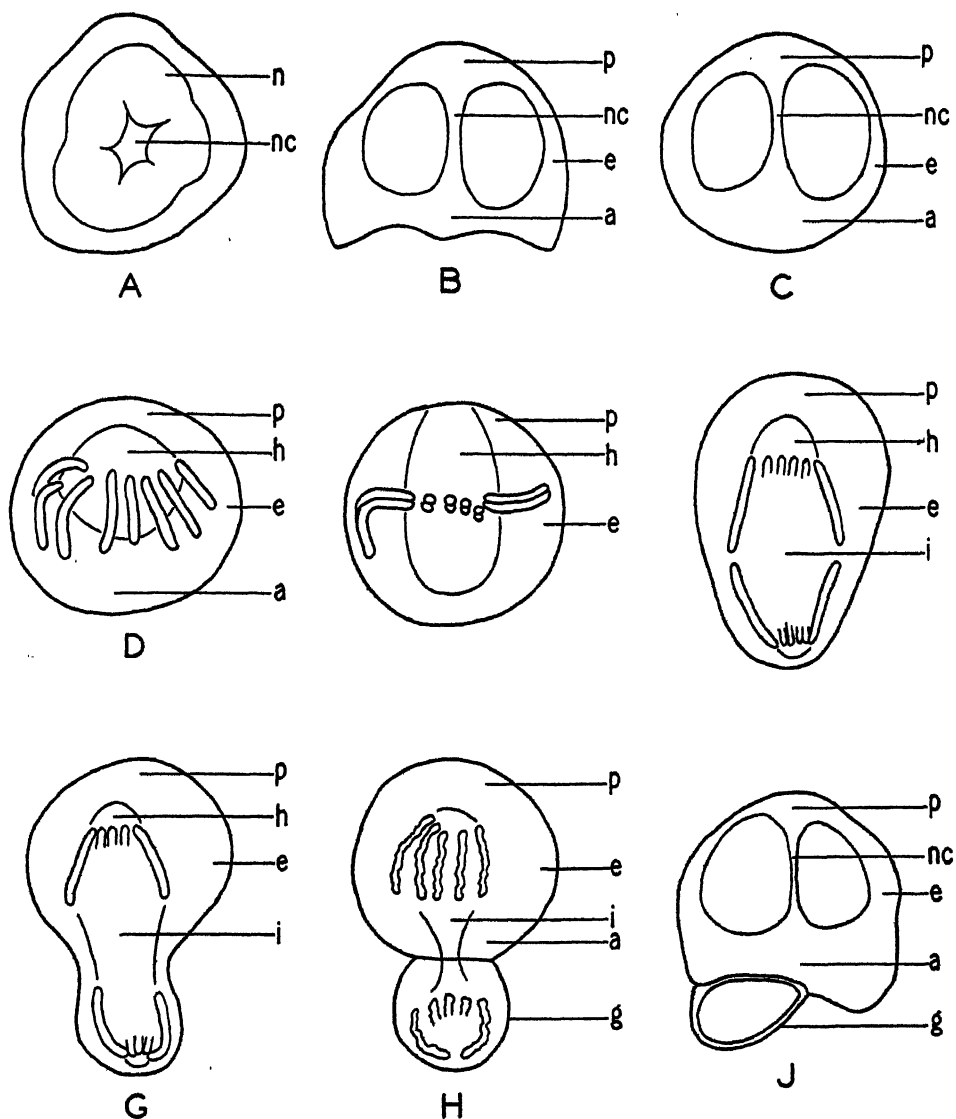


FIGURE 1. Representative stages in the mitotic cycle of the grasshopper neuroblast, reconstructed from camera lucida sketches of living cells growing in culture medium. *A* and *B*, polar and side views, respectively, of interphase or prophase cells; *C*, late prophase a few minutes before breakdown of the nuclear membrane, the cell having acquired a spherical form; *D*, prometaphase; *E*, metaphase; *F*, middle anaphase; *G*, late anaphase; *H*, early telophase; *J*, late telophase, the cell having lost its spherical form. *a*, polar cytoplasm; *e*, equatorial cytoplasm; *g*, ganglion cell; *h*, half spindle; *i*, interzonal region; *n*, nucleus; *nc*, nuclear core cytoplasm; *p*, polar cytoplasm.

viscosity values and the relative speeds of brownian movement are designated as follows:

Very high: No visible movement of mitochondria.

High: Movements very limited, discernible only with prolonged observation.

Medium: Movements readily observable, but not rapid.

Low: Movements quite free and rapid, but individual mitochondria can be followed with the eye.

Very low: Movements so rapid that individual mitochondria cannot be followed with the eye.

Even if the ability to make purely objective distinctions between adjacent members of this series be open to question, values separated by one class, viz., very high and medium, high and low, or medium and very low, can be distinguished readily and would give results essentially similar to those obtained.

Mitotic stages

The stages for which data were obtained and their distinguishing characteristics in the living, untreated state, when observed through 12.5 \times compensating oculars and a 2 mm. oil immersion objective, are:

Interphase: Nuclear threads appear in optical section like small, rounded, scattered granules in an otherwise homogeneous nuclear background. Period of growth.

Very early prophase: Nuclear granules smaller in size. Threads have begun to appear in the previously homogeneous background.

Early prophase: Nucleus filled with extremely fine chromatin threads. No nuclear granules.

Middle prophase: Chromatin threads of appreciable thickness. Ends when about seven chromosomes in cross-section can be counted in one-fourth the nuclear circumference.

Late prophase: Chromosomes well-formed. Toward the end of this period cell assumes spherical shape (Fig. 1B, 1C). Ends with the simultaneous disappearance of the nuclear membrane and appearance of the spindle.

Prometaphase (Fig. 1D): Chromosomes straighten and move into the equatorial plane. Spindle develops.

Metaphase (Fig. 1E): Chromosomes in equatorial plane.

Early anaphase: Begins with the initial separation of the chromatids and ends when the distal ends of the chromosomes leave the cell equator.

Middle anaphase (Fig. 1F): This stage is terminated as the cleavage furrow penetrates to the interzonal region.

Late anaphase (Fig. 1G): Ends as the chromosomes lose their sharp outlines and the cleavage furrow appears to be complete.

Early telophase (Fig. 1H): This stage lasts until the nucleoli become visible.

Middle telophase: The nucleoli increase in size while retaining their spherical shape, the nuclear membrane re-forms, the interzonal spindle remnant disappears, and the cell re-assumes the hemispherical form.

Late telophase (Fig. 1J) : Begins as the outlines of the nucleoli become irregular. Ends as linear arrangement of chromatin granules is lost.

The relative duration of these stages is given in an earlier paper (Carlson, 1941).

Regions of cell

Data have been obtained for six different regions of the cell, which are labeled in representative stages of the mitotic cycle in Figure 1.

Polar cytoplasm: This includes the region about the spindle pole of the daughter neuroblast when a spindle is present. At other stages it is the region within which the spindle pole of the daughter neuroblast will develop preparatory to the next division.

Apolar cytoplasm: The cytoplasm situated near the place of formation of the preceding cleavage furrow. It is opposite the polar region.

Equatorial cytoplasm: This is the portion of the cell that is situated at or near the cell equator in metaphase and anaphase and midway between the polar and apolar regions during the remainder of the mitotic cycle.

Nuclear core cytoplasm: The cytoplasmic core that passes through the nucleus (see p. 110).

Half spindle: This includes the developing spindle at prometaphase, the fully formed spindle at metaphase, and at anaphase the portion of the spindle lying between the pole and the plane in which the kinetochores of the adjacent chromosome group are situated.

Interzonal region: This is the part of the cell lying inside the ring of interzonal fibers and situated between the two separating groups of daughter chromosomes. It makes its appearance as the chromatids separate at early anaphase and persists as a spindle remnant into middle telophase.

Nucleus: No information that seemed reliable was secured for this part of the cell. Tiny nuclear granules comparable in size to the mitochondria showed very limited movement at various stages of the mitotic cycle. This might indicate a persistently high viscosity or it might mean merely that these granules are attached to the chromatin threads, which form a semi-rigid framework within the nucleus, and would therefore not be expected to exhibit much brownian movement. Bělář (1929a), however, has reported a rapid brownian movement of tiny granules of unknown nature situated in the ground substance of the nucleus of the grasshopper spermatocyte during prophase and telophase, indicating, according to him, that this substance is quite fluid.

OBSERVATIONS

The curves shown in Figure 2 were constructed in the following way. A linear series of arbitrary numerical values was assigned to the five selected viscosity classes described previously. Four observations were made for each region of the cell on different days and with different preparations. These were averaged and plotted. Because the determination of each of these was to a certain extent subjective, in that it depended on the judgment of the observer, and because the vis-

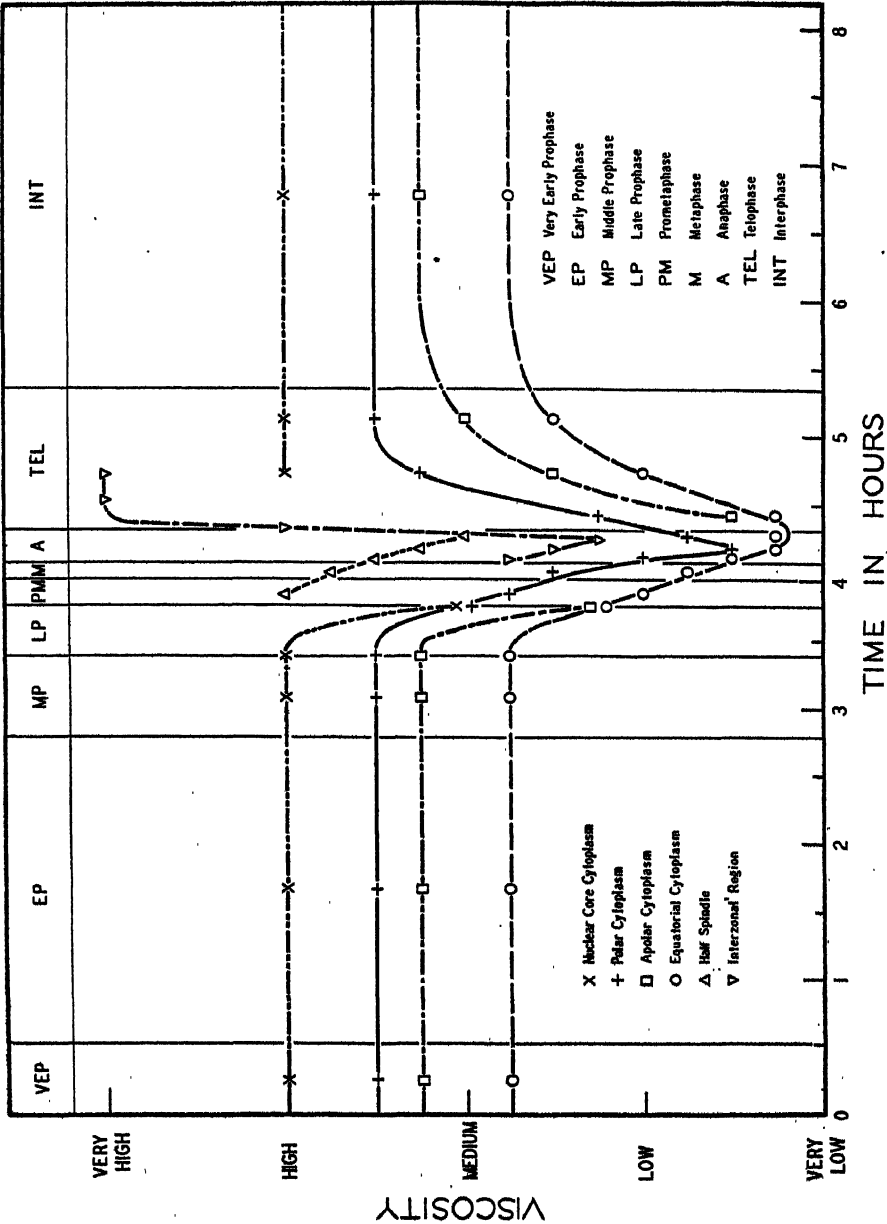


FIGURE 2

cosity varied slightly from preparation to preparation and from cell to cell, certain of these curves showed irregularities. In order to discover whether these were the result of experimental error or of actual viscosity shifts, new cultures were prepared, checked, and any necessary corrections made. It is believed that the graph in its present form represents quite accurately the cycle of viscosity changes in the different regions of the cell during mitosis. It should be emphasized, however, that the five arbitrarily-chosen viscosity values represented by ordinates are not necessarily a linear series of absolute values, as their graphical treatment implies.

During the greater portions of interphase and prophase the viscosity of the cytosome maintains a relatively high and constant value. In late prophase, a few minutes before the disappearance of the nuclear membrane, a rapid fall in viscosity is initiated. This change begins as the cell changes in shape from hemispherical (Fig. 1*B*) to spherical (Fig. 1*C*). The increase in the rapidity of mitochondrial movement is distinguishable first at the juncture of the equatorial and apolar regions as this part of the cell begins to draw in coincidentally with the start of the rounding-up process. From this region the lowered viscosity spreads into the apolar region, which thickens as the rounding-up proceeds, and subsequently into the equatorial and polar regions. Obviously, some of this is due to the actual movement of the cytoplasm, which must of necessity shift in position as the cell shape changes, but this can hardly be responsible for more than a small fraction of the viscosity change that takes place at this time. Since this alteration in shape occupies only about 5–10 minutes, this sequence of changes does not appear in the graph. The viscosity continues to fall through prometaphase and metaphase to reach a minimum in anaphase. The progress of the cell through telophase is accompanied by a steady rise in viscosity until the interphase-prophase level is attained. During telophase the hemispherical form of the cell is re-assumed (Fig. 1*J*).

The half spindle appears to show a slight fall in viscosity from prometaphase to early telophase, by which time it is too small for further study.

As the chromosome halves begin to separate at early anaphase, the interzonal region between the groups often contains one or more mitochondria showing a moderate amount of brownian movement. During middle and late anaphase large numbers of mitochondria move into the interzonal region, until they are as concentrated as outside, except for a small region inside each of the separating chromosome groups. Their movement is quite rapid, but less so than that of the mitochondria outside in the equatorial region. As the cleavage furrow presses in against the interzonal fibers, the transverse diameter of the interzonal region decreases and the motion of the mitochondria quickly slows down and then stops entirely. The fact that these stationary bodies are lined up in rows parallel with the long axis of the spindle makes it appear as if they had been caught among interzonal fibers of higher viscosity as these were pressed inward by the deepening cleavage furrow. By late telophase these fibers are no longer visible.

The high viscosity level of the nuclear core cytoplasm immediately after the formation of the nucleus and core may be due to the fact that this is the region occupied earlier by the spindle remnant of the preceding division.

DISCUSSION

The brownian movement method

Of the three most frequently used methods of making determinations of protoplasmic viscosity changes, viz., centrifuge, microdissection, and brownian movement, the last has the advantage of producing no physical disorientation of the cell contents. It can be depended on to give reliable results, however, only if certain factors are taken into consideration:

1) The protoplasm surrounding the granules in brownian movement must be homogeneous, if the viscosity of the protoplasm as a whole, exclusive of the granules, is being determined. If regions of different viscosity are present, observations of the rate of brownian movement of a particle will indicate only the viscosity of the material immediately surrounding it. The polar, equatorial, apolar, and nuclear core regions of the neuroblast cytosome are visibly homogeneous except for the mitochondria, and the fact that these bodies appear to migrate about and pass one another in the course of their shorter zig-zag movements demonstrates that they are not enclosed in a substance of one viscosity that is in turn surrounded by material of another viscosity. The spindle, however, is probably an exception to this (see p. 118).

2) Alterations in the size of the mitochondria from one stage of mitosis to another would cause an apparent shift in the viscosity of the surrounding medium, even though no actual change took place. Such changes could conceivably result either from shrinking and swelling as a consequence of osmotic shifts or from division and growth. The latter can be ruled out in the case of the neuroblast because very few mitochondria would need to divide in each cell generation to make up for the loss to the daughter ganglion cell, which receives very little cytoplasm and very few mitochondria. The resulting effect on brownian movement would be insufficient to alter the general viscosity picture. With regard to osmotic changes, Lewis and Lewis (1915) report that immersing cells in hypotonic solution causes swelling of the mitochondria. Bělař (1929a), on the other hand, states that mitochondria are particularly insensitive to the usual swelling effects of hypotonic solution. Unfortunately, the neuroblast mitochondria are so small and move so rapidly at certain stages that exact comparisons of size by actual measurements are impossible. Careful visual comparisons of the mitochondria of adjoining cells in different mitotic stages, however, have failed to reveal any significant size differences. As far as the present study is concerned, swelling of the mitochondria of late prophase, prometaphase, and metaphase cells by intake of water (p. 119) would tend to reduce rather than augment the observed viscosity shift in these stages; therefore, the actual change would be even greater than that shown in Figure 2.

3) If the granules under observation are so crowded as to interfere with each other's movements, the viscosity values obtained at the lower levels will be too high; for frequent collisions will retard their movements. The mitochondria of the neuroblast are definitely crowded, and this could easily result in appreciable errors in absolute viscosity values based on the migration of a given body a certain distance in a certain time, according to the method described by Pekarak (1930). Much of this error is doubtless avoided in the present study, however, because viscosity determinations were based on the rapidity with which the particles as a group dance about rather than on the total distance they migrate in a given interval of time.

Results of related studies

Of the several studies made on viscosity changes of the whole cytoplasm during mitosis, the centrifuge determinations of Heilbrunn (1917, 1921) for the marine invertebrate egg (Arbacia, Cumingia, and Nereis) and of Kostoff (1930) for meiotic cells of Nicotiana and the brownian movement determinations of Kato (1933) for the meiotic cells and staminate hairs of Tradescantia have given results that are similar to those I have obtained for the polar, apolar, and equatorial cytoplasm of the grasshopper neuroblast. All these studies show that the viscosity is relatively high during interphase, when interphase is referred to at all, high during all or most of prophase, falling during metaphase, lowest at anaphase, and rising at telophase.² The similarities in these results are all the more striking in view of the great diversity of the cell types used. The marine invertebrate egg contains relatively large quantities of ergastic matter, such as oil globules and yolk and pigment granules, and develops during mitosis an astral system and spindle that involve a large portion of the protoplast. The pollen mother cell, though consisting of a more or less homogeneous cytosome with relatively small spindle and no astral system, nevertheless is atypical mitotically because of the extended prophase and the two successive meiotic divisions without an intervening interphase. The staminate hairs of the plant contain large vacuoles with only a peripheral film and a few central strands of cytoplasm. The grasshopper neuroblast contains a fairly homogeneous cytosome with no visible ergastic matter or vacuoles but an abundance of mitochondria. The spindle is moderate in size and no asters are visible. This suggests strongly that viscosity change is a fundamental factor in mitosis, and is largely independent of individual peculiarities of the type of cell studied.

Zimmermann (1923), who studied the viscosity of the alga, Sphacelaria, by means of the centrifuge and brownian movement, and Seifriz (1920), whose studies of the marine invertebrate egg are based on microdissection, place the viscosity fall and minimum somewhat in advance of this mitotically. According to Zimmermann the viscosity is lowest at metaphase, while Seifriz finds it lowest during late prophase and metaphase. Both describe the viscosity as rising at anaphase and high at telophase.

In contrast with these findings are the results obtained by Chambers (1917, 1919) from studies of Arbacia and Echinarachnius eggs with the microdissection needle. He reported that the viscosity of the greater part of the cell is low during prophase, rising during metaphase, high during anaphase and early telophase, and falling in late telophase. He related the viscosity to the state of development of the amphiaster: low when no amphiaster is present, rising as the amphiaster forms, highest when it has reached its maximum development, and falling as the amphiaster disappears at the end of mitosis. He does, however, describe a liquefaction of the equatorial region previous to anaphase and persisting through cleavage.

Fry and Parkes (1934) duplicated as closely as possible the centrifuge studies of Heilbrunn on Arbacia, Cumingia, and Nereis eggs. The results they obtained for viscosity in relation to time after fertilization were identical with those reported earlier by Heilbrunn; in fact, in their paper they used Heilbrunn's curves for Arbacia and Nereis eggs. They interpreted this data, however, as supporting

² Heilbrunn associates the pre-cleavage fall and the post-cleavage rise in viscosity of the Arbacia egg with spindle development and disappearance, respectively.

Chambers' conclusions, claiming that Heilbrunn had misidentified certain of the mitotic stages. This would place viscosity changes of the marine invertebrate egg in entire disagreement with those of the grasshopper neuroblast, in which there can be no question of the correct identification of the different mitotic stages.

Kostoff's description of viscosity changes in somatic cells of *Nicotiana* (1930) is not in accord with any of these results. Using the centrifuge technique, he reported two cycles of viscosity change for each mitotic cycle: high viscosity at prophase and anaphase, and low viscosity at metaphase and interphase.

Observations on the viscosity of the spindle are complicated by the probable presence in the half spindle of two materials, spindle fibers and interfibrillar substance. If there are two such materials present, the mitochondria or other cytoplasmic granules that make their way into the spindle by brownian movement would be expected to occupy the region of lower viscosity, i.e., the interfibrillar region. Under such conditions their speed of movement would be determined by two factors: the viscosity of the interfibrillar substance and the amount of space available for movement between the fibers, especially if this were very limited.

Bělař (1929b) found that most spindles in cells of the stamen hairs of *Tradescantia* contained a few tiny granules in quite rapid brownian movement. This motion of the granules, which appeared first in the polar caps (clear regions that adjoin opposite sides of the nucleus at late prophase and in which the spindle later develops), was evident in the fully-formed spindle and continued undiminished up to the time of formation of the cell plate, when the granules disappeared from view. This contrasted with the situation he found in the spindles of animal cells, namely, nematode eggs, lepidopteran and grasshopper spermatocytes, and Actinophrys, in which granules of a comparable size in the spindle were always relatively quiet (see Bělař, 1929a). He interpreted this difference to indicate that in the *Tradescantia* cell there was a larger amount of the less viscous interfibrillar substance than in the animal cells he studied. I have not been able to confirm Bělař's observation that the granules show greater freedom of movement in the direction of the long axis of the spindle than at right angles to it, but this may mean only that the interfibrillar substance is more abundant and the fibers farther apart in my material than in his.

Ris (1943) reports unrestricted brownian movement of the cytoplasmic granules that make their way into the interzonal region during anaphase in embryonic cells of *Tanalia*. Chambers (1924) states that the spindle of the dividing sand-dollar egg has become "distinctly fluid" by the time the chromosomes have reached the poles. He doubtless refers to what I have termed the interzonal region. These observations agree well with the conditions in the grasshopper neuroblast.

Possible causal factors in viscosity change

With regard to the factors responsible for viscosity changes of the cytoplasm, two possibilities seem deserving of consideration: alterations in the water content and in the pentose nucleic (ribonucleic) acid content of the cytoplasm.

It has already been pointed out (p. 115) that the first detectable viscosity drop coincides with the initiation of the "rounding-up" of the neuroblast in late prophase and that the viscosity is rising as the cell flattens on one side to assume the hemispherical form during telophase. Since it can be demonstrated that immersion of

these cells in hypotonic culture medium causes a fall in viscosity owing to intake of water and that hypertonic medium causes a rise in viscosity through loss of water, it seems not unlikely that the late prophase viscosity fall and "rounding-up" of the cell might result from the intake of water, while the telophase viscosity rise and the accompanying flattening of the cell at one side might be due to loss of water. Unfortunately, the irregularities in the hemispherical-shaped cell make it impossible to determine whether there is any actual change in cell volume during the alteration in shape. Chambers (1919) relates the change in form of the *Arbacia* egg from spherical to hemispherical following the first cleavage division to a viscosity shift, but he associates it with a lowering of the viscosity.

Water exchange seems inadequate, however, as a complete explanation of the observed viscosity changes for two reasons. First, it seems doubtful that the intake of water would occur in sufficient amounts to account entirely for the observed viscosity fall. Second, the viscosity continues to rise for some time after the cell has returned to its hemispherical shape, when the water loss might be supposed to have been completed. Another factor, therefore, would appear to be involved, either in place of or in addition to water intake and loss.

In support of the possibility that changes in the pentosenucleic acid content of the cytoplasm may be at least partly responsible for the observed viscosity changes is the fact that pentosenucleic acid, which is known to be present in large quantities in the cytoplasm of rapidly growing tissues (Brachet, 1933; Caspersson and Schultz, 1940) and which has a strikingly high viscosity ($\eta = 62.4$, according to Cohen and Stanley, 1942), undergoes a change in amount per cell during mitosis (Brachet, 1940; Caspersson, 1940; Painter, 1943). Both Brachet, from studies of the eggs and early developmental stages of different animals, and Painter, using *Rheo* meiotic cells, reached the conclusion that cytoplasmic pentosenucleic acid is abundant in mitotically active cells at early prophase, less abundant or entirely absent from late prophase through anaphase, and increasing in amount following division. They believe that the late prophase decrease is due to transformation of pentosenucleic acid into the desoxypentosenucleic acid of the developing chromosomes and that the increase following division is due to the loss of desoxypentosenucleic acid from the chromosomes and its transformation into pentosenucleic acid. These changes in the nucleic acid content of the cytoplasm during mitosis bear a very close resemblance to my viscosity curve for the neuroblast cytoplasm. If there is any discrepancy, it would seem to be the exact time during late prophase that the nucleic acid leaves the cytoplasm in detectable amounts, and neither the studies of Brachet nor Painter furnish information on this point. The statement of White (1942), however, that "the nucleic acid of the chromosomes undergoes a sudden increase at prometaphase, when the nuclear membrane breaks down and substances from the cytoplasm have free access to the chromosomes" suggests that the fall in cytoplasmic nucleic acids may coincide closely with the viscosity fall of the cytoplasm. The viscosity rise, which extends through all of telophase, could be accounted for, up to the time of nuclear membrane formation, by a return of the pentosenucleic acids to the cytoplasm during the retrogressive chromosome changes, and, after re-constitution of the nuclear membrane, by the synthesis of new pentosenucleic acid.

SUMMARY

Observations on the rapidity of brownian movement of the mitochondria in different regions of the grasshopper neuroblast during the entire mitotic cycle indicate that:

1) The viscosity of all parts of the cytosome is relatively high during interphase and prophase, begins to fall in late prophase, reaches a minimum at anaphase, and rises gradually to its original high level during telophase.

2) The viscosity of the portion of the spindle between the pole and the plane in which the proximal ends of the chromosomes are situated appears to fall slightly from a high prometaphase level through anaphase.

3) The viscosity of the portion of the spindle situated between the separating daughter chromosome groups shows, during anaphase, a slight drop from a medium value, and this is followed at the beginning of telophase by an abrupt rise to a very high level, which is maintained through early and middle telophase.

Alterations in water content and nucleic acid content of the cytoplasm are suggested as possible explanations of viscosity changes during mitosis.

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TOXIC EFFECTS OF COPPER ON ATTACHMENT AND GROWTH OF *BUGULA NERITINA*¹

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INTRODUCTION

This paper describes physiological experiments dealing with the effect of copper on attachment and early development of *Bugula neritina* (L.), a widely distributed marine fouling organism. The studies were designed to gain a clearer understanding of the specific role of copper in prevention of fouling and the mechanism of action of copper paint surfaces. Various experiments using copper solutions and paints were made to gain an integrated concept of copper toxicity.

Copper, in one form or another, has been used since ancient times to prevent fouling and is one of the most effective metallic poisons for marine fouling organisms (Visser, 1927). The antifouling efficiency of metallic poisons is related to the intrinsic toxicity of their ions and the solubility of their corrosion products in sea water, both relatively high for copper (Parker, 1924). Recently, this concept has been clarified and extended by Ketchum, Ferry, Redfield, and Burns (1945) who show that the prevention of fouling is related to the concentration of toxic dissolved in the water at the surface of the paint, and that the rate of loss of toxic is a measure of the steady-state concentration in a narrow zone at the paint surface. The rate of loss of toxic from a paint surface is known as the leaching rate. The authors show that in the case of copper paints, a leaching rate maintained consistently above a minimum of 10 micrograms of copper per square centimeter per day will prevent serious fouling.

Much discussion and some experimentation has centered around the question of the relative toxicities of different forms of copper. Carritt and Riley (1943), however, were unable to demonstrate any difference in toxicity to *Bugula turrita* between cuprous and cupric ions, while Clarke (1943) found that "all forms of copper have roughly the same toxicity but differ greatly in their solubilities."

The establishment of fouling organisms on a submerged surface depends *a priori* on their ability to affix themselves and to develop after attachment. Effective antifouling surfaces, therefore, must function in one or both of two ways, by repelling (or killing) larvae of fouling organisms, or by inhibiting development of any that attach. Visser (1927), Neu (1932), and Edmondson and Ingram (1939)

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conclude from indirect evidence obtained mainly in field studies that toxic paints have no effect on growth of fouling organisms once attached, that they function only by repelling or delaying attachment. Without minimizing the repellent action of antifouling paints, evidence is accumulating to show that toxic surfaces function importantly through inhibition of growth of attached larvae. The ingenious experiments of Pomerat and Weiss (1943) showing inhibition of fouling growth on unpainted areas by adjacent toxic paint surfaces strongly support this view. Similar "distance effects" of antifouling paint surfaces on fouling growth on adjacent untreated areas are frequently seen in panel tests (e.g., Edmondson, 1944, Fig. 7a, p. 13).

Toxic effects of copper on various fouling organisms have been described by Bray (1924), Whedon *et al.* (1942 and 1943), Miller and Cupp (1942), Clarke (1943), and Riley (1943) in unpublished reports to the Bureau of Ships, U. S. Navy Department. Prytherch (1934) reports stimulating effects of small amounts of copper on settling and metamorphosis of the oyster, and similar oligodynamic effects on ascidians were observed by Grave and Nicholl (1939).

Jones (1941) reviews theories on the mechanism of toxic action of metals, notably copper. These may be divided into two main groups which maintain that: (1) copper retards vital processes through inactivation of essential enzymes, and (2) copper acts more directly by precipitating cytoplasmic proteins as copper-proteins. With regard to fouling organisms, Clarke's (1943) studies on barnacles and mussels, and Riley's (1943) investigations and those of the present author on bryozoans favor the inactivation theory. Further studies are needed, however, to elucidate the physiological mechanisms involved.

The results of the present investigation will be presented in four parts: (1) attachment and growth of *Bugula* on copper paint surfaces, (2) growth of *Bugula* in copper solutions, (3) recovery of *Bugula* from copper poisoning, and (4) toxicity gradients of copper paint surfaces.

MATERIALS AND METHODS

Bugula larvae for these studies were obtained as follows. Mature colonies were collected from docks, piling, etc., in San Diego Bay the day before tests were made, and placed in aquaria of sea water in the laboratory. Early the next morning, these liberated swarms of larvae which were easily collected at the light side of the container since they are positively phototropic and visible to the naked eye (about 0.2 mm. in length and darkly colored). *Bugula* larvae normally attach and begin to grow before mid-afternoon of the day on which they are liberated as described by Grave (1930) and Edmondson (1944). In subtropical localities, as San Diego Bay, larvae may be obtained the year around. From the foregoing facts, it is obvious that this form is remarkably suited for studies on fouling problems.

Primed steel, 1 x 3 inch panels were coated with the various experimental and control paints employed in the first section of this paper. Larger panels (10 x 12 inches) were similarly prepared and exposed in the bay to test their antifouling efficiency under field conditions. In most cases, the small panels were "seasoned" before testing by soaking them for a month or more in large jars of sea water. The seasoning bath was changed at frequent intervals and aerated by means of a stream of air bubbles delivered at the base of the container by means of a tapered glass tube.

For other studies, larvae were allowed to attach to small test panels which were coated with a non-toxic paint composed of equal parts of paraffin and ester gum. So attached, they could conveniently be transferred to the experimental situations or be removed therefrom for observations.

Other procedures will be described as necessary in the various sections of the investigation.

Attachment and growth of Bugula on copper paint surfaces

The first problem was to determine the effect of actual copper paint surfaces on attachment and growth of *Bugula* larvae under simulated natural conditions. The data given are typical of many such tests that have been performed.

TABLE I

Attachment and growth of Bugula larvae on hot and cold plastic paints, each series with graded copper content. (Paints seasoned for two months in sea water bath before tests were made.)

Series	Paint No.	Percentage Cu ₂ O in dry paint	Leaching rate (2-mo.) μg Cu/cm. ² /day		Per cent larval attachment ± S.E.		Growth (3-day) Maximum length ¹ (mm.)	
			Filmed	Clean	Filmed	Clean	Filmed	Clean
Hot plastic	1	37.0	28.1	24.1	4±1.6	2±1.1	0.3	—
	2	32.0	19.4	23.7	23±5.3	15±4.4	0.3	0.3
	3	26.0	14.0	14.0	5±3.3	13±6.3	0.2	0.2
	4	19.0	13.5	11.5	79±3.5	72±5.0	0.2	0.2
	5	10.6	6.9	5.8	86±4.4	95±2.6	0.4	0.6
	6	0	—	—	100	100	1.3	1.2
Cold plastic	7	36.0	14.3	14.1	0	3±2.4	—	0.2
	8	30.0	14.1	13.3	5±3.0	1±1.4	0.2	0.2
	9	19.2	14.1	13.0	7±2.4	10±4.5	0.3	0.2
	10	9.3	3.5	3.8	87±3.6	73±6.8	0.6	0.7
	11	0	—	—	78±7.4	98±1.4	1.1	1.1

¹ As attached larvae are approximately 0.2 mm. in length, maximum growth increments may be calculated by subtracting this value from the maximum lengths given above.

Two series of copper paints (one hot plastic and one cold plastic series)² were prepared, each having the same matrix composition but graded amounts of cuprous oxide (Table I). Two test panels were coated with each of these paints and seasoned for ten weeks. Prior to testing, one panel in each duplicate set was wiped to remove the slime film that accumulated during seasoning; the other was used without being cleaned.

At the start of the attachment test, the panels were immersed in jars containing 700 cc. fresh sea water and immediately thereafter a known number of *Bugula* larvae (usually 100) were added. Tests were always started in mid-morning when the larvae were active, and counts of the number attached were made late in the after-

² A hot plastic paint is one that is applied hot and forms a solid film on cooling; cold plastic paints are one of the types of paints which dry by evaporation of the solvent. The paints used in these experiments were experimental modifications of standard Navy formulations.

noon when all larvae were either attached or dead. The percentage of attachment was calculated by dividing the number of larvae affixed to the test panel by the total number of larvae used.

Data on development, which involves both growth and differentiation, were obtained by measuring the length from base to apex of the young *Bugula* stalks, and by determining the number of polypides developed from time to time. Length measurements were made with an ocular micrometer under low magnification.

The results of the attachment and growth tests on the various copper paint surfaces are given in Table I and in Figures 1 and 2. The data clearly segregate two groups of paints. Those with less than 15 per cent Cu_2O content or leaching rates less than 10 micrograms of copper per square centimeter per day are obviously inferior as over 70 per cent of the larvae were able to attach to them and to grow significantly. These paints foul in a more or less short period of time in field tests. The second group, with greater copper content and concomitantly higher leaching rates, were significantly more efficient in repelling attachment and permitted no significant growth. These paints are more effective in the field, the length of their effective periods being related to their copper content.

That leaching rate rather than copper content is responsible for the antifouling performance of a paint can easily be demonstrated by comparing *Bugula* attachment and growth on surfaces with identical amounts of cuprous oxide but different leaching rates. If, for example, ester gum is substituted on an equal weight basis for rosin as the resinous component in our hot plastic series, leaching rates fall far below the adequate level and *Bugula* larvae attach abundantly and develop colonies on such surfaces. Ketchum *et al.* (1945) have shown that substitution of ester gum, esterified Albortol or coumarone-indene for the rosin content of an effective antifouling paint (Navy Department Specification 52-P-161) drastically reduces the leaching rate below the critical level with the result that such paints foul quickly in the field. Thus, copper content is important only insofar as it contributes to an adequate and sustained leaching rate, and may be neglected in the subsequent discussion.

In these tests, the slime film on the various paint surfaces had no consistent effect on larval attachment. Its presence apparently is not necessary for attachment of *Bugula* since larvae affixed themselves to cleaned panels as well as those with a coating of slime film, and since they readily attach themselves to unseasoned surfaces shortly after their initial submergence and certainly before any visible film forms. Whether or not an invisible film is prerequisite to attachment is an academic question. In the tests cited, the larvae had no choice between surfaces with and those without slime film. When such choice is involved, *Bugula* larvae do exhibit certain preferences (Whedon *et al.*, 1943). The role of the slime film on attachment to toxic and non-toxic surfaces under various experimental conditions will be considered in another paper.

Larval attachment to paints with leaching rates above 15 micrograms of copper per sq. cm. per day ranged between zero and 23 per cent with an average of less than 10 per cent. None of these was able to grow, however, and hence to establish a colony. The range of leaching rates between 10 and 15 $\mu\text{g.}$ per sq. cm. per day may be regarded as a "marginal zone," especially as the lower value is approached, since paints with leaching rates in this range often permit considerable attachment

of larvae. These may or may not be killed. If not, as will be shown later, some of them might recover and grow should the leaching rate and subsequent surface concentration of toxic fall below the adequate growth inhibiting level. Even if the attached larvae are killed, however, there is still the possibility that their dead bodies might form a less toxic substrate for later larvae than the paint surface

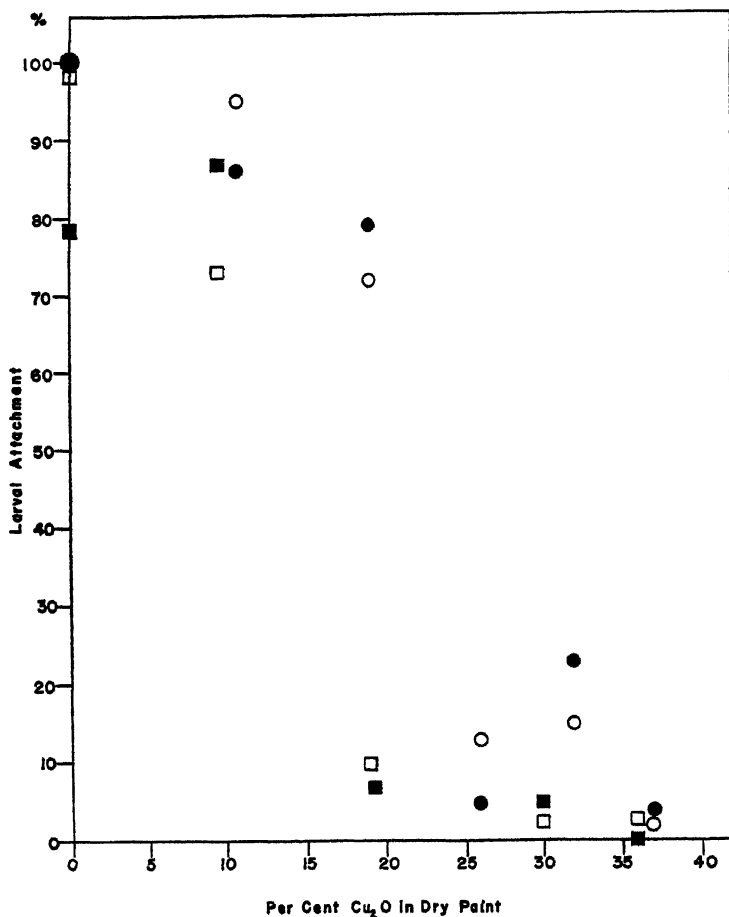


FIGURE 1. Attachment of *Bugula* larvae in relation to copper content of paint. Circles represent hot plastic paints while squares indicate cold plastic paints. Black-filled data points represent paint surfaces coated with slime film while clear data points represent cleaned surfaces (wiped before testing). All paints were seasoned for ten weeks in sea water before testing.

itself. Larvae frequently attach themselves on top of other larvae, often in clusters. Conceivably, those underneath might take up the toxic ions diffusing from the paint or in other ways shield the overlying larvae so that the latter could develop. Certainly, those on top would be further removed from the paint surface and hence would be located in a less concentrated part of the toxic zone which might permit

their development. While this sort of attachment is obviously not very secure, firmer attachment may eventuate by means of stolon-like outgrowths from developing colonies to the paint surface. Such root-like processes are developed by erect bryozoans and may give rise to secondary colonies.

The fact that at least a small percentage of larvae attach at any one time to quite toxic surfaces indicates that under field conditions the growth inhibiting func-

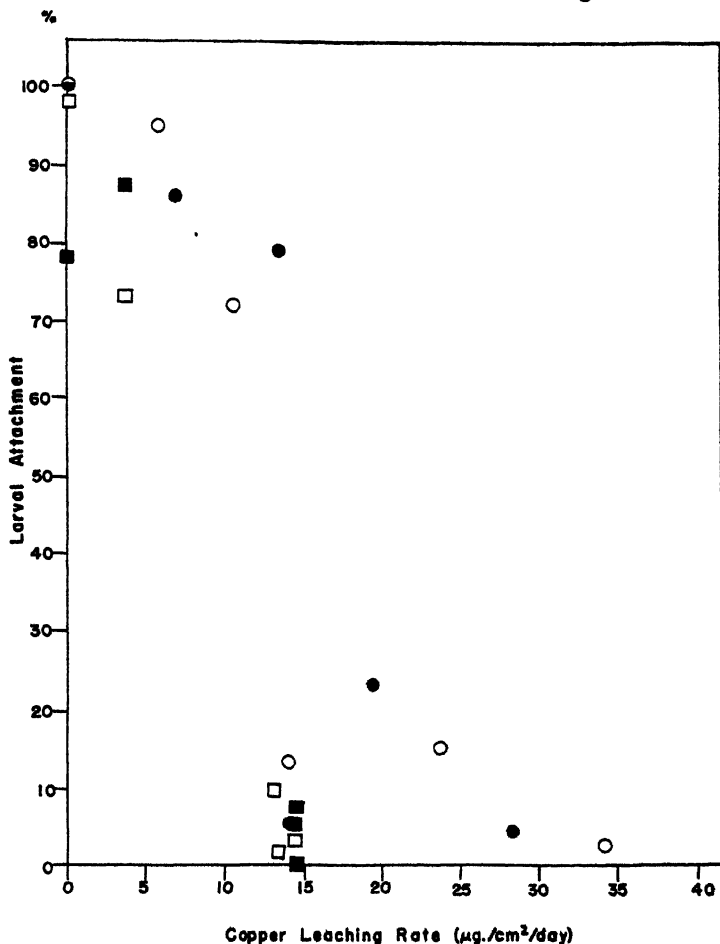


FIGURE 2 Attachment of *Bugula* larvae in relation to copper leaching rate. Symbols as in Figure 1.

tion of toxic surfaces plays an important role in the prevention of fouling. Effective antifouling surfaces must function in part by stopping development of attached larvae. This is contrary to the previously mentioned views of Visscher, Neu, and Edmondson and Ingram. The latter authors report a restricted growth of *Bugula* and serpulid worms on toxic surfaces exposed in Hawaiian waters, but attribute this to "delayed attachment rather than to a slower rate of growth after settling."

Dates of attachment in their tests are not known, and hence rates of growth on treated and untreated surfaces cannot be calculated and compared from their data. In view of the present studies, it would seem that the alternate interpretation of a growth retarding effect is equally plausible, if not more probable.

To illustrate further the growth retarding effect of toxic surfaces, another experiment involving growth of *Bugula* on unseasoned copper paint surfaces may be cited. In this experiment, the first two weeks of growth of *Bugula* on two hot-plastic, copper-paint surfaces (*A* and *B*) was compared to that on a non-toxic, hot-plastic control (*C*). The two toxic paints have identical copper content (32 per cent

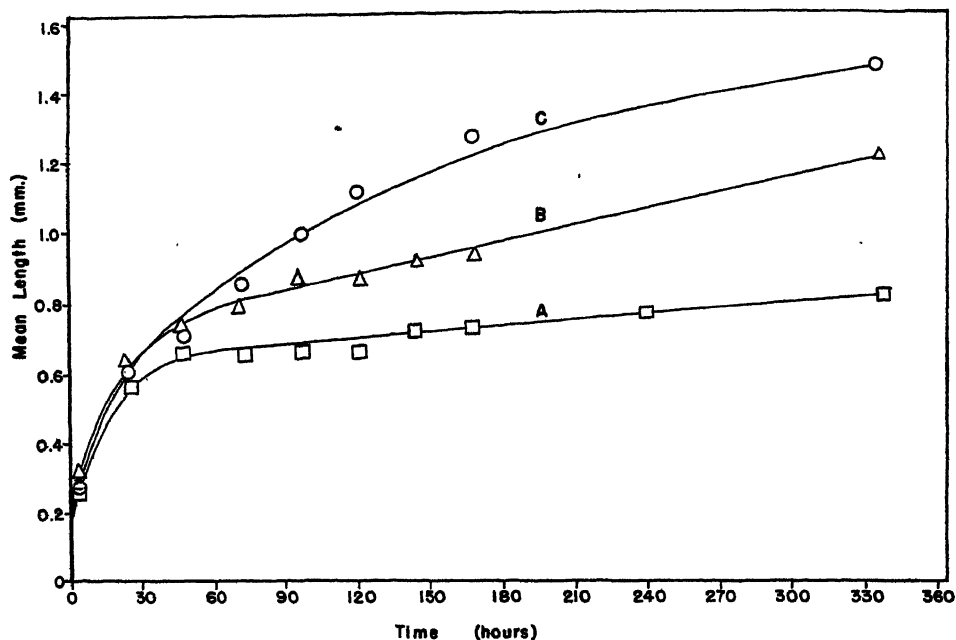


FIGURE 3. Growth of *Bugula* ancestrulae on unseasoned hot-plastic paints. *A* is an excellent copper paint with adequate leaching rate, *B* is an inferior paint with low copper leaching rate, and *C* is a non-toxic control.

Cu_2O), but paint *A* (w.w. rosin-paraffin matrix) has an excellent field record correlated with adequate leaching rates, while paint *B* (ester gum-paraffin matrix) fouls quickly in the field because of low leaching rate. Hot-plastic paints of the type here used have low initial leaching rates and do not develop characteristic leaching rates until they have been submerged for a period of time. Hence, *Bugula* larvae attach abundantly to unseasoned panels coated with these paints and begin to grow normally on them.

As shown in Figure 3, the growth curves in the present experiment do not begin to diverge significantly until after the second day. Growth on paint surface *A* was practically stopped at this time at the first polypide stage. Some individuals developed the first polypide, most did not, and no colonies were established. Colonies were developed on paint surface *B*, but growth and differentiation were sig-

nificantly less than for controls. The three curves become more and more divergent with time.

The foregoing differences between growth inhibiting properties of paints *A* and *B* must be attributed to differences in their leaching rates as their copper content and growth of *Bugula* on their respective controls are identical. Repeated leaching rate determinations of these paints have shown both to have low initial leaching rates (which accounts for the initial similarities in the *Bugula* growth curves for the two surfaces) but paint *A* eventually develops an adequate leaching rate, while leaching rates of paint *B* do not attain the adequate level. Thus, again the growth retarding property of copper paint surfaces is demonstrated and associated with the copper leaching rate.

TABLE II
Effect of copper on growth and development of Bugula ancestrulae

Av. copper concentration mg./liter	Growth and differentiation in copper solutions			Polypides per colony	
	Mean length increments \pm S.E.				
	1 day	3 days	5 days	3 days	5 days
	mm.	mm.	mm.		
Controls	0.36 \pm .007	0.61 \pm .016	0.77 \pm .021	1-2	1-2
0-.05	0.30 \pm .015	0.55 \pm .013	0.63 \pm .018	1	1-2
0.05-.10	0.26 \pm .009	0.37 \pm .010	0.46 \pm .012	1	1-2
0.10-.15	0.25 \pm .007	0.36 \pm .008	0.48 \pm .013	1	1
0.15-.20	0.14 \pm .008	0.22 \pm .009	0.24 \pm .011	0-1	0-1
0.20-.25	0.08 \pm .007	0.10 \pm .008	0.12 \pm .010	0	0
0.25-.30	0.02 \pm .007	0.03 \pm .008	0.02 \pm .009	0	0
0.30-.35	0.04 \pm .011	0.03 \pm .011	0.03 \pm .009	0	0
0.35-.40	-0.03 \pm .008	0.01 \pm .009	0 \pm .008	0	0
0.40-.45	0.03 \pm .007	0.02 \pm .011	-0.03 \pm .010	0	0
0.45-.50	0.02 \pm .008	0.03 \pm .010	0.03 \pm .010	0	0

The experiments cited in this section are in close accord with results obtained by Ketchum *et al.* (1945) and substantiate the leaching rate theory of action of anti-fouling paints. It is noteworthy that their estimate of minimum adequate copper leaching rate (10 micrograms per sq. cm. per day), which was based on comparisons of numerous leaching rate determinations with field exposure tests, is confirmed by the *Bugula* attachment and growth tests. Data presented in this section show that copper paints with leaching rates less than this value permit larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 10 micrograms per sq. cm. per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms of copper per sq. cm. per day, but these do not develop into colonies.

Growth of Bugula in copper solutions

To determine more precisely the effect of copper ions on growth of *Bugula*, non-toxic panels bearing attached larvae (about 25) were immersed in sea-water solutions of graded copper concentrations. Cuprous oxide was the salt used in making

the copper solutions, but the ions were presumably cupric since the oxidation of cuprous ion is supposedly rapid. The young *Bugulae* were measured just before immersion in the experimental solutions and at one, three, and five day intervals thereafter. Polypide development was also observed at these times. Mean length increments after immersion were computed as an index of toxicity of the solutions.

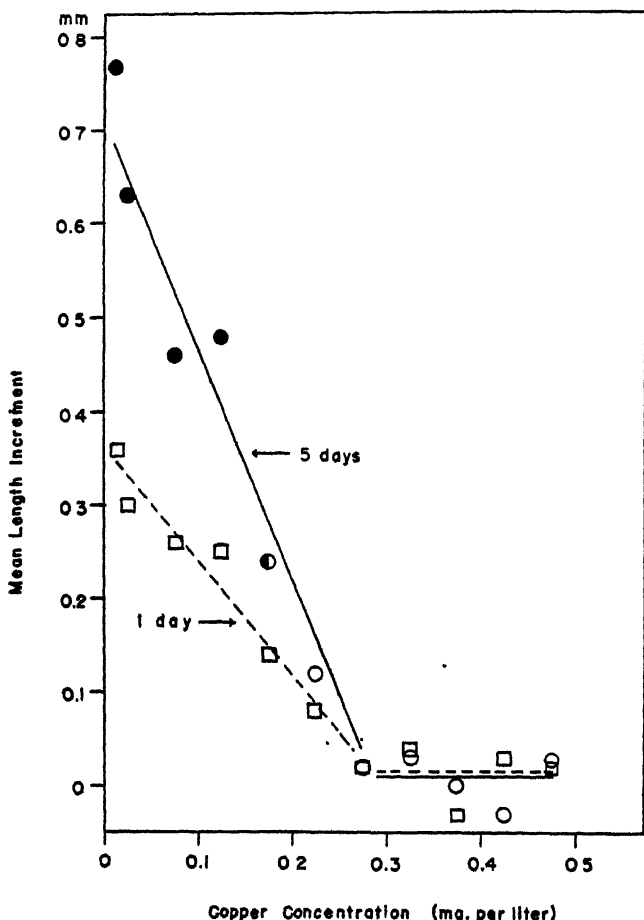


FIGURE 4. Growth of *Bugula ancestrulae* in relation to copper concentration. Curves for one-day (squares) and five-day increments (circles) based on grouped data (class intervals = 0.05 mg. copper per liter). Filled squares indicate one or more polypides per colony, half filled squares show that some stalks did not differentiate polypides, and open data points indicate that no polypides were developed. See also Figure 5.

The copper concentrations of each solution were determined before and after the experiment, and the average values were used.

As clearly shown in Table II and Figures 4 and 5, the degree of early growth and differentiation of *Bugula* is closely related to the copper concentration in the water surrounding the organisms. Up to about 0.3 mg. per liter, increment in

length is inversely proportional to copper concentration of the solution. Higher concentrations inhibit growth completely or allow but slight, insignificant increment. The sharp break or inflection in the curves in Figures 4 and 5 at about 0.3 mg. copper per liter apparently marks this as a critical concentration for growth. No stimulating effect of small amounts of copper on growth was noted, since even in the greatest dilutions used growth increments were less (with minor exceptions) than in fresh sea-water controls.

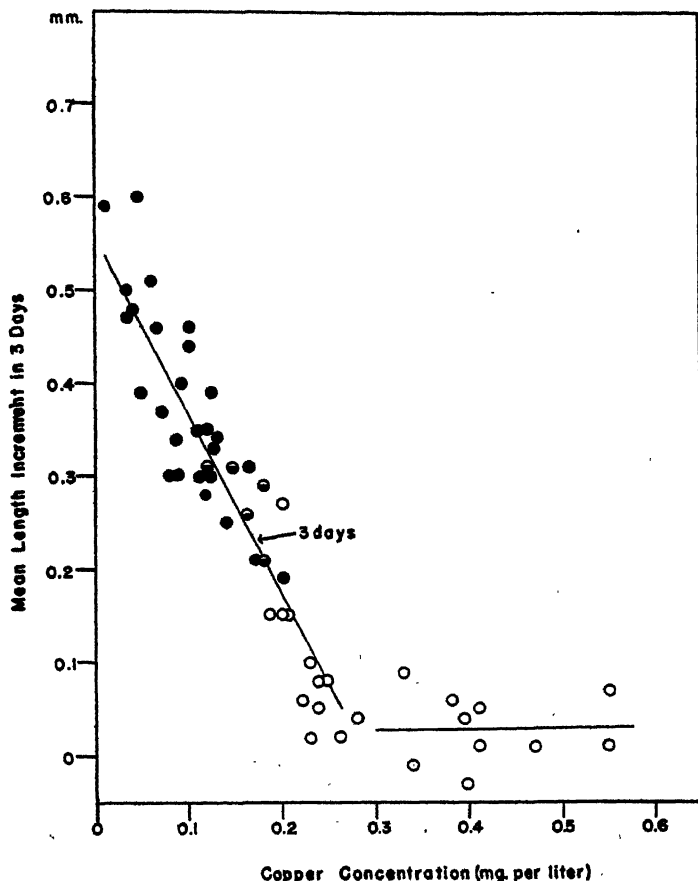


FIGURE 5. Three day growth of *Bugula ancestrulae* in relation to copper concentration. Black circles indicate fully developed polypides, half filled data points represent partly formed polypides, and clear circles indicate no differentiation of polypides. See also Figure 4.

Polypide differentiation is inhibited by copper concentrations greater than 0.2 mg. per liter, the minimal value being less than that required to prevent growth completely. Concentrations less than 0.2 mg. per liter only delay polypide development. This is shown by the fact that only controls attained the second polypide stage at three days (Table II), by the several cases of incomplete or non-functional polypides at three and five days in the range between 0.1 and 0.2 mg.

per liter (Figs. 4 and 5), and by the fact that at five days only the first polypide was developed in concentrations greater than 0.1 mg. per liter.

A series of critical copper concentrations may be postulated from these and other studies (Table III). The minimum lethal dose (MLD) for free swimming larvae is about 0.3 mg. per liter (Miller and Cupp, 1942), the same concentration required to stop growth of attached larvae. No data are available on concentrations necessary to prevent larval attachment. Presumably these would be at least as great as the minimum lethal dose, and probably greater, since larvae in solutions containing as much as 0.4 to 0.5 mg. copper per liter do not die immediately but swim about more and more slowly for a half hour to an hour or more. Given a suitable surface, it is conceivable that some might attach in this interval.

TABLE III
Critical copper concentrations

Copper concentration (mg. per liter)	Physiological effect
>0.3	Kills larvae. Inhibits growth.
0.2-0.3	Retards growth. Inhibits polypide development.
<0.2	Retards growth and development of polypides.

Recovery of Bugula from copper poisoning

The question arises, are the above-described toxic effects of copper permanent, or can organisms recover to any significant degree after exposures to sublethal dosages? Preliminary experiments showed that *Bugula ancestrulae* can recover nearly normal development after immersion for 6 to 24 hours in sublethal copper sea-water solutions (Miller and Cupp, 1942). In this experiment, the periods of immersion in copper solutions were extended (three to seven days) and alternated with periods of immersion in fresh sea water to determine the effect on recovery of longer exposures and of intermittent as compared to continuous dosages.

The initial procedure was similar to that of preceding experiments. A non-toxic test panel with attached larvae was immersed in each of eight copper sea-water solutions (Nos. 1-8, Table IV) for a period of three days (time period I). The subsequent procedure features a staggered schedule of transfers of the animals from copper solutions to fresh sea water, so that, while one group was exposed to copper, the other was immersed in untreated sea water and *vice versa*. After the first three days exposure, half of the panels (group A, sets 1-4) were transferred to fresh sea water, and the remainder (group B, sets 5-8) were left in the original copper solutions. After four days (period II), group A panels were transferred from sea water back to the original copper solutions, while group B sets (that had now been in the toxic solutions for a week) were transferred to fresh sea water. After another four days (period III), the process was again reversed—those in sea water were transferred back to copper solutions and *vice versa*. The experiment was terminated on the fifteenth day, the final four days constituting period IV.

Length measurements were made before the original immersion (a few hours after attachment of the larvae), at the time of transfers (on the 3d, 7th, and 11th days), and at the termination of the test on the 15th day. From these data, the rates of growth relative to that of controls for the four time periods in copper solutions and sea water were computed. Concentrations of the copper solutions

were estimated from their biological effects (using curves in Figs. 4 and 5) as chemical determinations were not made for this test. Data are given in Table IV, and the salient features are shown in Figure 6.

In all but one case, transfers to fresh sea water were followed by marked and significant increases in growth rate and polypide differentiation, while the reciprocal transfer resulted in sharply decreased, if not completely inhibited, growth (Fig. 6). The exceptional case (set 7) presumably received a lethal dose in the first period as no significant increases in length and certainly no differentiation were observed following transfer to fresh water. All others, however, exhibited an amazingly high degree of plasticity and regulatory ability in recovering from the effects of the

TABLE IV

Growth and development of Bugula ancestrulae during and after immersions in copper sea-water solutions

Symbol No.	Cu. in solns. mg./liter	Mean length increments, time periods I-IV*				Total no. of polypides per colony at end of periods:			
		I (3 days) mm.	II (3-7 days) mm.	III (7-11 days) mm.	IV (11-15 days) mm.	I	II	III	IV
A-1	0.26	0.05	0.34	0	0.14	0	1	1	2
-2	0.27	0.04	0.22	0.01	0.09	0	1	1	2
-3	0.24	0.09	0.27	0.10	0.14	0	1	1	2
-4	0.23	0.11	0.25	0.09	0.06	0	1	1	2
B-5	0.25	0.08	0.01	0.12	-0.02	0	0	1 ab.	1
-6	0.23	0.10	0.01	0.17	-0.02	0	0	1 ab.	1
-7	0.3	0.03	0.03	0.05	0	0	0	0	0
-8	0.20(?)	0.14	0.16	0.23	0.10	1	1	1	2
Control		0.68 (0.68)	0.64 (0.53)	0.11 (0.28)	0.25 (0.19)	1	2	3	4

* Group A immersed in copper solutions during periods I and III, and in fresh sea water during periods II and IV. Group B immersed in copper solutions during periods, I, II, and IV, and in fresh water during period III.

poison. Even those immersed an entire week in solutions that allowed but slight growth increment and no visible differentiation (e.g., sets 5 and 6) were able to recover significantly, from growth rates of zero to as much as 60 per cent that of controls, when restored in fresh sea water. They also differentiated polypides though these were slightly abnormal in some instances. Set 8 in group B was apparently exposed to a weaker solution than any of the others since these individuals not only grew considerably but also developed polypides even while immersed in the copper solution. Nevertheless, significant increases and decreases in relative growth rates for this set were observed in the various periods. Seemingly, one could control rate of growth and differentiation almost at will by appropriate exposures to copper. The ability of young *Bugulae* to recover each time after repeated and long immersions in rather toxic copper solutions is truly remarkable.

In no case was recovery complete as judged either by growth rates or polypide development. Growth rates after transfers to sea water ranged roughly between a third and four-fifths of the normal (see Fig. 6), and the number of polypides developed was less than that of controls

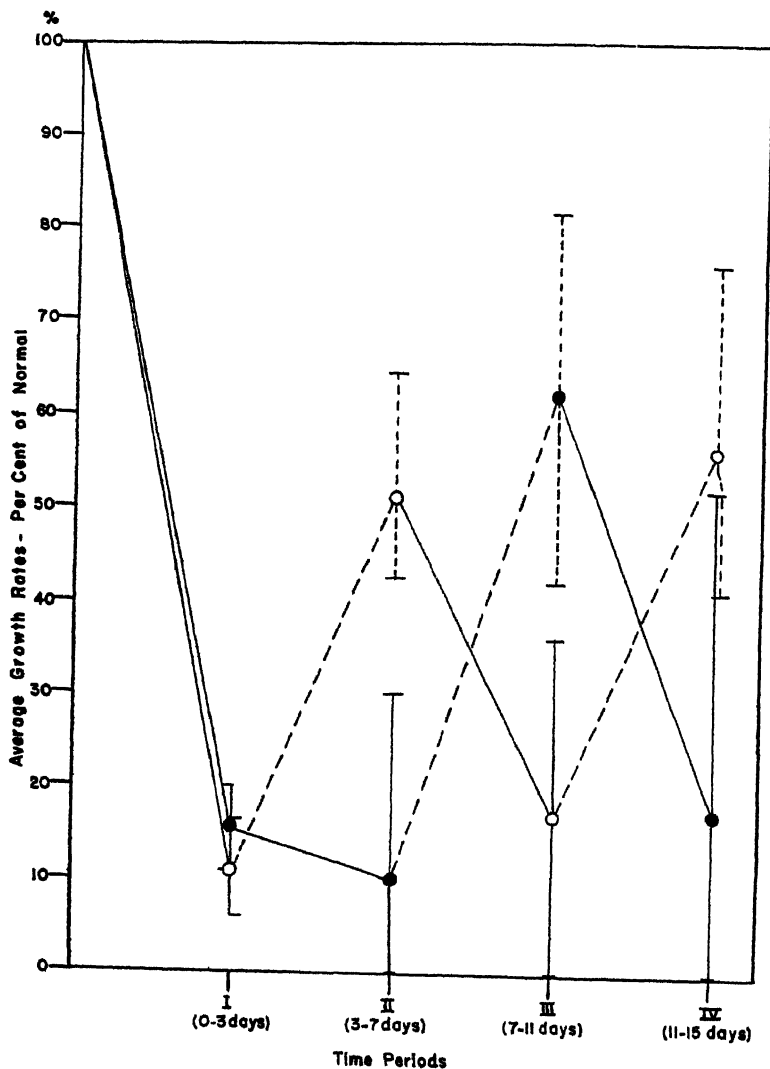


FIGURE 6. Growth rates of *Bugula ancestrulae* (in per cent of normal) during and after exposure to sublethal copper concentrations. Black data points represent averages for group A panels (Table IV), and clear circles are averages for group B panels (omitting set 7). Solid lines indicate periods of immersion in copper solutions, and dashed lines represent transfer to fresh sea water. Vertical lines through data points show the range between minimum and maximum growth rates for each average

Length of exposure in sublethal concentrations has relatively little apparent effect on recovery. This is shown by the fact that *Bugulae* exposed to copper for seven days were able to develop after transfer to sea water at a rate comparable to that of specimens which had been exposed for only three days. As previously noted, however, the longer exposures caused abnormal polypide development in some cases.

In contrast to this finding, there is evidence that in the case of barnacles and mussels the length of exposure is as important as the concentration in determining the toxic effects of copper solutions. Clarke (1943) found that low concentrations acting for a long time produced effects equivalent to high concentration acting for a short time. In other words, the toxic effect is proportional to the product of duration and intensity. Further experiments of this general type are needed to determine more precisely immediate and long-term effects of various exposures and concentrations and of continuous versus discontinuous exposures.

These experiments have practical implications since, under natural conditions, the concentration of toxic ions adjacent to a copper paint surface might vary from time to time as a result of fluctuation in leaching rate, formation of surface films, currents flowing past the surface, and other factors. The effect of such changes on attached *Bugula* larvae might be surmised from the foregoing studies showing that growth is an inverse function of copper concentration in the medium and that stunted individuals can resume development when restored to non-toxic situations. Presumably, *Bugula* ancestrulae attached to a surface with fluctuating toxicity might recover to some degree with each significant decrease, if the organisms were still viable. With each increase in length, the ancestrulae greatly improve their chances to establish colonies for reasons which will become apparent in the next section of this paper.

Toxicity gradients of copper paint surfaces

The question of zones or gradients of toxicity adjoining toxic paint surfaces is involved in understanding their antifouling action, and is the last problem to be considered in this paper. Although a toxicity gradient has been assumed as a consequence of diffusion of toxic ions emanating from an antifouling paint, a demonstration of this seemed desirable. Furthermore, information on the effective limits and other characteristics of the toxic zone is of particular interest in connection with the establishment of colonial fouling organisms (e.g., erect bryozoans and most hydroids) that grow, plant-like, more or less perpendicularly away from the surface to which they attach, and develop new individuals at the ends of their branches. It would be useful to know how far from the toxic surface growth inhibiting and growth retarding concentrations are maintained, or how much a colony would have to grow before its terminal polypides were out of danger from poisoning. In the following experiment, the problem was to demonstrate, if possible, the existence of the toxic zone of an antifouling paint and to determine its general characteristics.

The preceding studies, showing rather precise relationships between growth of *Bugula* ancestrulae and copper concentration, suggested a method for attacking the problem of toxic gradients. The essential features of the procedure used and of the results obtained are illustrated in Figure 7. A non-toxic panel bearing at-

tached and growing *Bugulae* was placed perpendicularly against a panel coated with a cold-plastic copper-paint (Table I, No. 7). With this arrangement, the developing *Bugula* stalks maintain practically constant distances between their axes and the toxic surface since they grow parallel to the latter. The non-toxic panel was ruled in millimeter divisions paralleling the toxic surface, and these were used as class intervals of distance in analyzing the data. The ancestrulae in each division were measured at the start of the tests and two days afterwards to determine the effect of diffusing copper ions on their growth at various distances from the paint surface. For control, a non-toxic panel was substituted for the toxic panel. Experimental and control racks were placed in an aquarium containing ten liters of sea water. The water was aerated during the tests by fine streams of air bubbles delivered through pinholes in a piece of rubber tubing stretched along the bottom of the aquarium. This method of aeration caused some circulation of water but no appreciable agitation.

TABLE V

Toxicity gradients of a copper paint demonstrated by growth of Bugula at measured distances from the toxic surface

Distance from paint surface	Test 1 Mean growth increment \pm S.E. (2 days)		Test 2 Mean growth increment \pm S.E. (2 days)	
	Experimental	Control	Experimental	Control
mm.	mm.	mm.	mm.	mm.
0-1	0.10 \pm .02	0.48 \pm .02	0.01 \pm .01	0.43 \pm .02
1-2	0.27 \pm .03	0.42 \pm .05	0.03 \pm .01	0.46 \pm .02
2-3	0.40 \pm .06	—	0.16 \pm .03	0.44 \pm .03
3-4	0.38 \pm .06	0.50	0.30 \pm .06	0.49 \pm .02
4-5	0.35 \pm .10	0.50	0.40	0.50 \pm .04
5-6	0.45 \pm .08	0.45 \pm .04	0.37 \pm .04	0.50
6-7	0.55 \pm .04	0.45 \pm .04	0.35	—
7-8	0.45 \pm .08	0.40	0.45	0.40
8-9	0.50 \pm .07	0.50	0.35 \pm .04	0.40
9-10	0.50	0.43 \pm .03	0.41 \pm .02	0.50

Two tests were made: the first, using toxic panels that had been seasoned for ten weeks in the laboratory; the second, with the same panels that were seasoned another two weeks. In the first tests, the slime film which had accumulated during seasoning was not removed but for the second tests the slime was wiped off. Data are given in Table V and graphically illustrated in Figures 7 and 8.

As clearly demonstrated by the retarded growth of the colonies near the painted surface, the toxicity arising from the surface decreases rapidly with distance from the surface. The outer effective limit of the toxic zone is variable. Beyond two millimeters from the surface in the first test and four millimeters in the second, no significant difference in growth between experimentals and controls was demonstrated. These values, then, represent the respective outer limits of the toxic gradient in the two tests and indicate the order of magnitude of the width of the toxic zone for a good antifouling paint. Within these zones, growth increments are roughly proportional to perpendicular distance from the toxic surface as might be expected from the diffusion gradient of toxic ions. Ancestrulae immediately

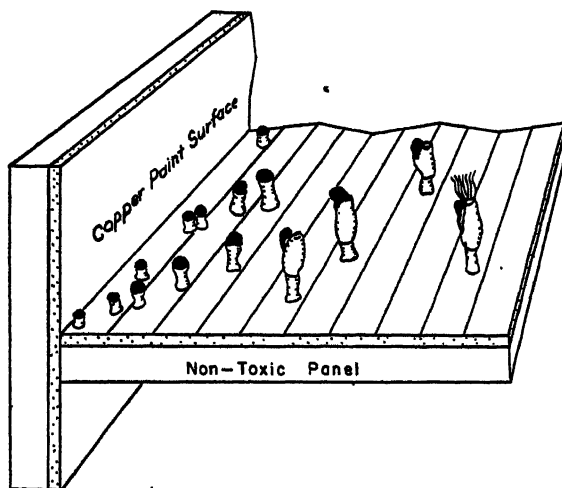


FIGURE 7. The toxic gradient extending from a copper paint surface as shown by growth of *Bugula ancestrulae* at measured distances from the toxic surface. *Bugula* figures are camera lucida drawings made four days after start of test 2 (for two-day growth, see Table V), and are twice enlarged in comparison to the millimeter rulings shown on the non-toxic panel.

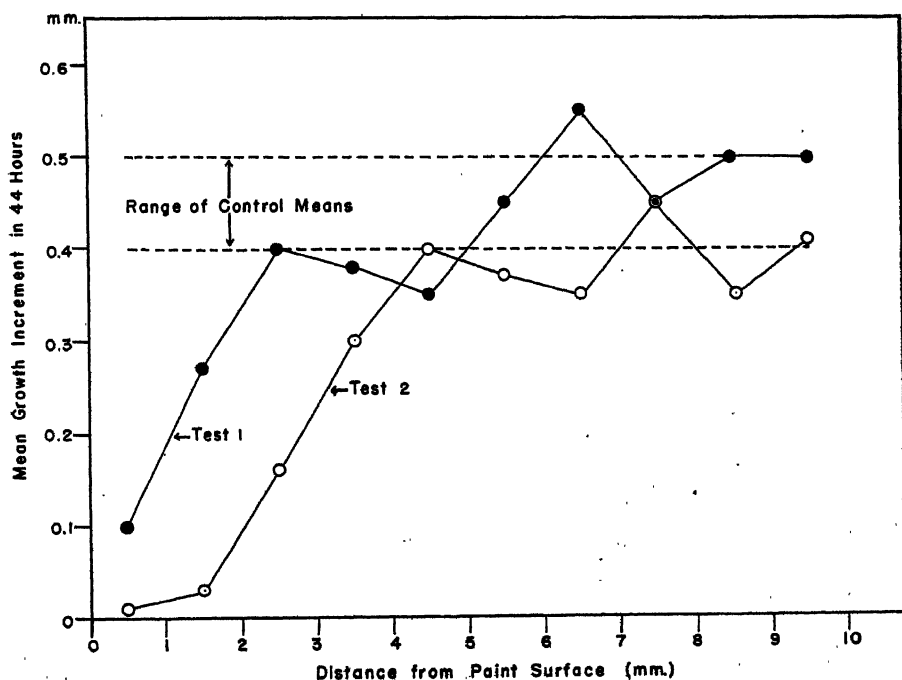


FIGURE 8. Gradients of toxicity of an antifouling paint: test 1—paint seasoned for ten weeks and coated with slime film, and test 2—after twelve weeks seasoning and with slime film removed before testing.

adjacent to the toxic paint, however, showed no increase in length indicating that growth inhibiting concentrations of copper were maintained there. This result was anticipated since larvae attached to surfaces coated with this paint do not grow (Table I, No. 7). Growth within the first millimeter interval is contributed by ancestrulae in the outer part of it. The width of the growth inhibiting portion of the toxic gradient (practically, the most important part of it) could be more precisely determined by further tests using smaller intervals of distance.

The panels used in the second test were clearly much more toxic than those first tested, as shown by the greater width of the toxic zone, by the greater growth inhibition near the surface, and by the fact that the curve of growth increments at distances greater than four millimeters tends to fall below that of controls. With respect to the latter point, the mean growth increment for all individuals between four and ten millimeters in test two is significantly less than the corresponding mean in test one or that for controls. The means for single millimeter intervals in this range are not significantly different probably because of the small numbers of individuals in each.

The characteristics of the toxic zone are undoubtedly affected by various factors such as leaching rate and velocity of flow of water across the surface. In the field, currents or movement of the painted surface through the water would probably alter the character of the toxic zone. This and other factors could be simulated in the laboratory and their effects analyzed using the above-illustrated methods with appropriate modifications.

The demonstrated vertical gradient would probably differ from a horizontal gradient that extends outward from the edge of a painted surface. The latter type was nicely demonstrated by Pomerat and Weiss (1943) in field tests with panels on which areas of various shapes and sizes were left unpainted. The horizontal gradient observed by these authors was expressed both by graded growth of fouling on large unpainted areas, and by absence of fouling on smaller areas encircled by the paint. Their effects might be attributed in part to delayed larval attachment as well as retarded growth since the settling of larvae cannot be controlled in the field. For practical purposes, the activity of a vertical gradient is probably of greater importance.

The foregoing experiment together with those reported in preceding sections of this paper clearly indicates that the prevention of *Bugula* fouling on copper paint surfaces is dependent upon their ability to maintain growth inhibiting concentrations of copper in a narrow zone at the surface. The length of newly attached *Bugula neritina* larvae (about 0.2 mm.) presumably represents the minimum adequate width of the growth inhibiting zone required to prevent establishment of this organism. If attached forms are permitted to grow, their apical developing parts move away from the toxic surface and hence into regions of lower toxicity with consequent acceleration of development. As new polypides are produced by distal budding, they find themselves in a less toxic environment than their predecessors and eventually the terminal polypides would lie entirely outside of the toxic zone. Since the polypides are functionally independent (except for support), the colony can flourish even though its basal members are dead. If the toxic zone extends outward only a few millimeters, just a few of the basal polypides would be affected since *Bugula* colonies at the first polypide stage average about 0.9 mm. in length and each successive polypide adds at least a half millimeter to the length.

Larvae that settle on previously attached forms or on any inert particles elevated appreciably above the surface plane of the paint would clearly stand a better chance of survival than those attached directly to the toxic surface. They would occupy less toxic regions of the toxic gradient which might permit their growth, while those attached to the surface itself might be killed or permanently stunted by the higher concentration of the toxic prevailing there. Judging from the steep slopes of the toxicity gradients for an effective paint (Fig. 8), a fraction of a millimeter from the paint surface might make a significant difference in development, especially on surfaces with borderline toxicity.

To summarize: the foregoing preliminary tests clearly demonstrate a zone or gradient of toxicity that extends outward a few millimeters from an effective copper paint surface. Further experiments, using the method illustrated, are indicated to determine more precisely the limits and other characteristics of the toxic zone under various conditions.

SUMMARY

Copper paint surfaces prevent the establishment of *Bugula neritina* (1) by repelling or killing the larvae and (2) by inhibiting growth and metamorphosis of attached larvae.

Copper paints with leaching rates less than 10 micrograms of copper per square centimeter per day permit the larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 15 micrograms per square centimeter per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms per square centimeter per day, but these do not develop colonies.

No consistent effect of slime film on larval attachment was noted. Its presence is not prerequisite to attachment.

Precise relationships between copper concentration and growth of *Bugula ancestrulae* are demonstrated. Growth in sea-water solutions of copper is inversely proportional to the concentration up to 0.3 mg. per liter. Higher concentrations completely inhibit growth.

The critical copper concentrations affecting various stages of the early life cycle of *Bugula* are as follows: (1) Concentrations greater than 0.3 mg. per liter kill larvae and completely inhibit growth of attached forms, (2) concentrations between 0.2 and 0.3 mg. per liter retard growth and prevent polypide formation, and (3) concentrations less than 0.2 mg. per liter retard growth and polypide development.

No stimulation of growth by copper solutions was observed. There was some evidence that small concentrations of copper stimulated attachment of larvae.

Bugula ancestrulae can recover and develop almost normally after seven days exposure to sublethal concentrations of copper. They can recover after repeated immersions in copper solutions that practically prevent growth. Length of exposure has relatively little effect on their ability to recover from copper poisoning.

A gradient of toxicity extending outward a few millimeters from a copper paint surface is demonstrated.

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A NEW GRAPHIC METHOD OF DESCRIBING THE GROWTH OF ANIMALS

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Growth curves, when conventionally plotted as length on age, are difficult to compare and classify. Moreover, the usual mathematical methods of fitting them, such as, the logistic and the Gompertz are rather laborious and inconvenient for application to large numbers of individuals.

Fortunately, for many purposes, it is unnecessary to describe the whole growth curve; for the part below the inflection point is completed early and the part above the inflection point—the “self-inhibiting” phase, covers the period of life when differences in growth are likely to be most striking. That phase of the growth curve can be appropriately represented by a straight line, the characteristics of which can be treated statistically, by the following graphic method:

Using arithmetic graph paper, with body length represented along both the x axis and along the y axis, plot length at ages 1, 2, 3, 4, 5 $\cdots n$ on the x axis against length at ages 2, 3, 4, 5, 6 $\cdots n + 1$, respectively, on the y axis. For several species on which I have found published length data, these points fall along a straight line. This line can be regarded as a sort of *transformation* of the usual growth curve, and in the following discussion I will call it that.

The nine examples given in Figures 1–3 are based on average lengths of large samples. When lengths of individual specimens are plotted by this method, a straight-line relationship is still obvious, though the points deviate more widely from the line than when averages are used. These deviations doubtless result from several causes, among which random variations in environmental experience and errors of observation readily suggest themselves. For a few species the published growth data failed to produce a straight line. In these cases, the course of growth may differ from that in other animals; or the observed anomalies may reflect some artifactual effect in the data.

Among those species for which this “transformation” results in a straight line, the growth increments corresponding to equal time intervals, for example, between years of age ($l_2 - l_1$, $l_3 - l_2$, $l_4 - l_3$, $\cdots l_n - l_{n-1}$), have the following interrelations; where l_n refers to the length at any given age, i.e., at the end of any given time interval:²

$$\frac{l_3 - l_2}{l_2 - l_1} = \frac{l_4 - l_3}{l_3 - l_2} = \frac{l_5 - l_4}{l_4 - l_3} \cdots = \frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$$

¹ For advice and assistance in the mathematics of this paper, I am indebted to Professors George Polya and Harold Bacon of Stanford University. I am also grateful to my colleagues, Mr. O. Elton Sette and Dr. Frances Felin, for their constant interest and help.

² In this discussion, l_1 is not necessarily the value directly obtained by measurement, but a value calculated on the basis of all measurements (see Fig. 4).

OR

$$l_n - l_{n-1} = k (l_{n-1} - l_{n-2}). \quad (1)$$

It is interesting to note that, with $l_0 = 0$,

$$\begin{aligned} l_2 - l_1 &= k (l_1 - l_0) = k l_1 \\ l_3 - l_2 &= k (l_2 - l_1) = k k l_1 = k^2 l_1 \\ l_4 - l_3 &= k (l_3 - l_2) = k k^2 l_1 = k^3 l_1 \\ l_5 - l_4 &= k (l_4 - l_3) = k k^3 l_1 = k^4 l_1 \\ &\vdots \\ l_n - l_{n-1} &= k (l_{n-1} - l_{n-2}) = k k^{n-2} l_1 = k^{n-1} l_1. \end{aligned} \quad (2)$$

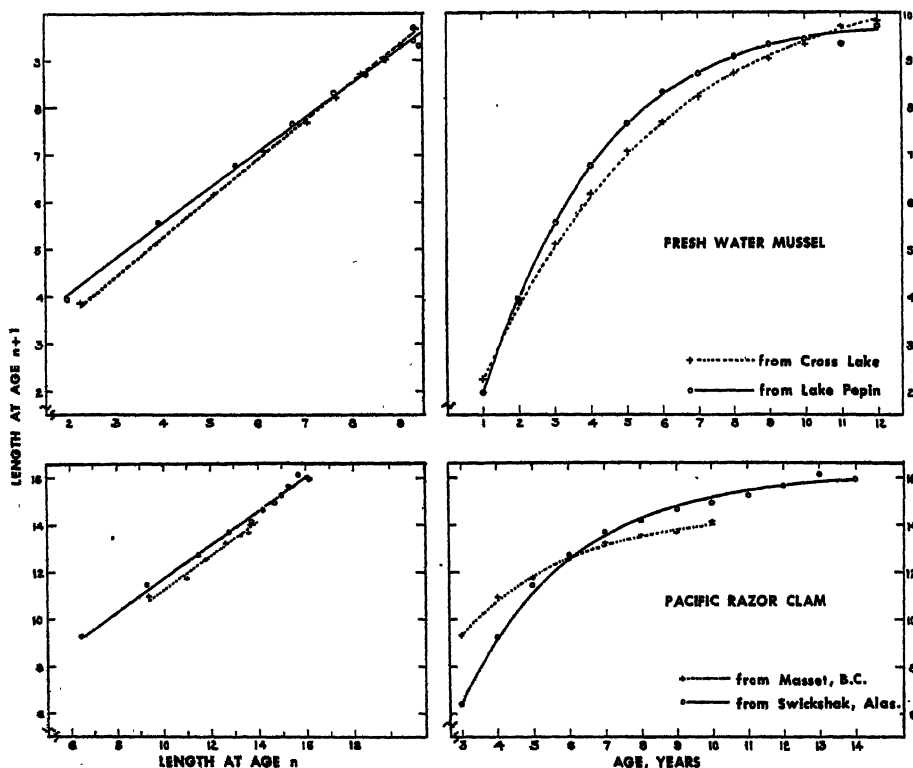


FIGURE 1. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on fresh water mussel from Chamberlain (1931); on Pacific razor clam from Weymouth (1931).

The constant k is positive and less than one; that is, the yearly growth increments decrease.

These relationships are consistent with there being a growth capacity, which is approached from the inflection point at a constant percentage rate. This is in accord with interpretations made by a number of students of growth, among

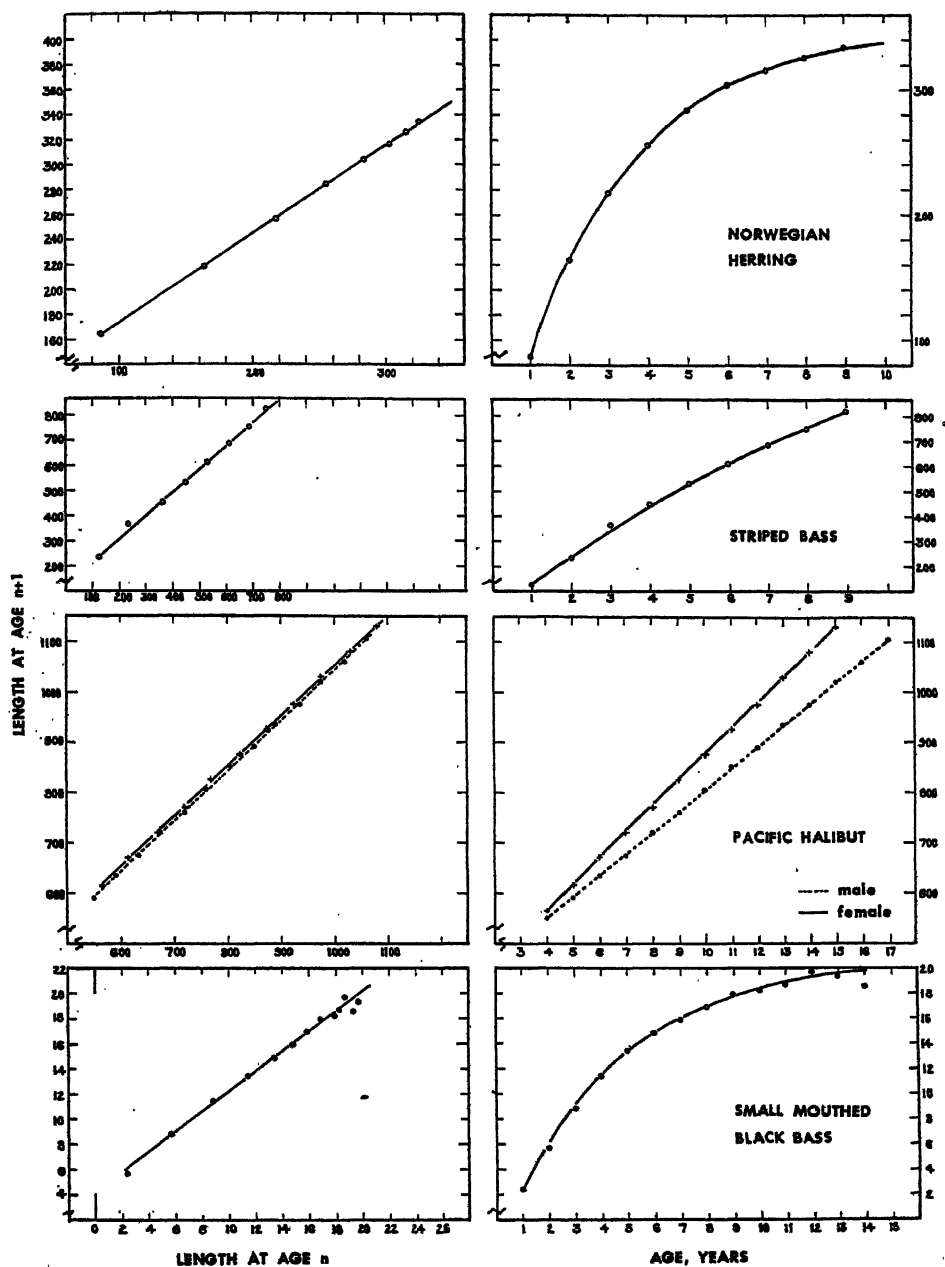


FIGURE 2. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on Norwegian herring from Runnström (1936); on striped bass from Merriman (1941); on Pacific halibut from Thompson and Bell (1934); on small mouthed black bass from Bennett (1938).

them Minot (1908), Wright (1926), Brody (1927 a, b, c), and Weymouth, McMillan, and Rich (1931).

The length at infinite age, l_{∞} , which can be regarded as the ultimate length or limiting length, can be calculated as follows: The length l_n is attained by adding to l_1 the successive increments,

$$l_n = l_1 + (l_2 - l_1) + (l_3 - l_2) + (l_4 - l_3) + \cdots + (l_n - l_{n-1}).$$

Yet these increments were expressed in the formulas following (1), thus:

$$l_2 - l_1 = k l_1, l_3 - l_2 = k^2 l_1, \cdots l_n - l_{n-1} = k^{n-1} l_1. \quad (3)$$

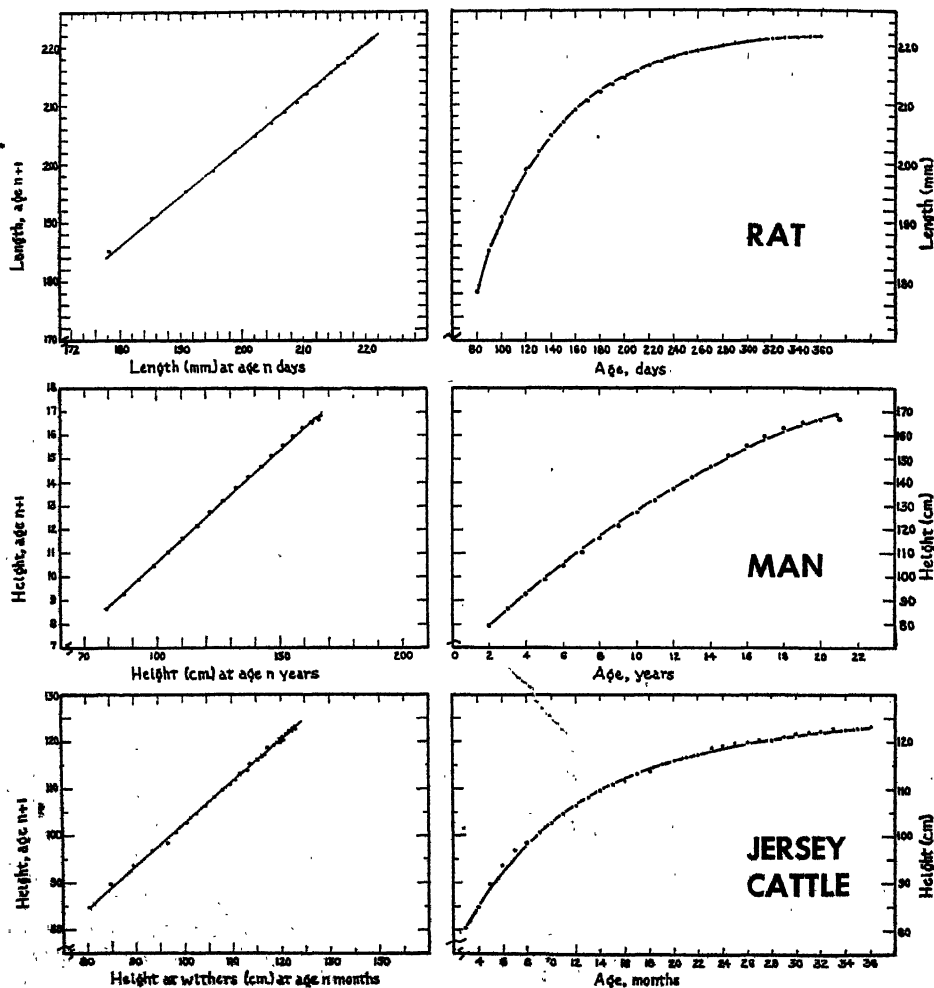


FIGURE 3. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on rat from Donaldson (1931); on man from Thompson (1942); on Jersey cattle from Ragsdale, Elting, and Brody (1926).

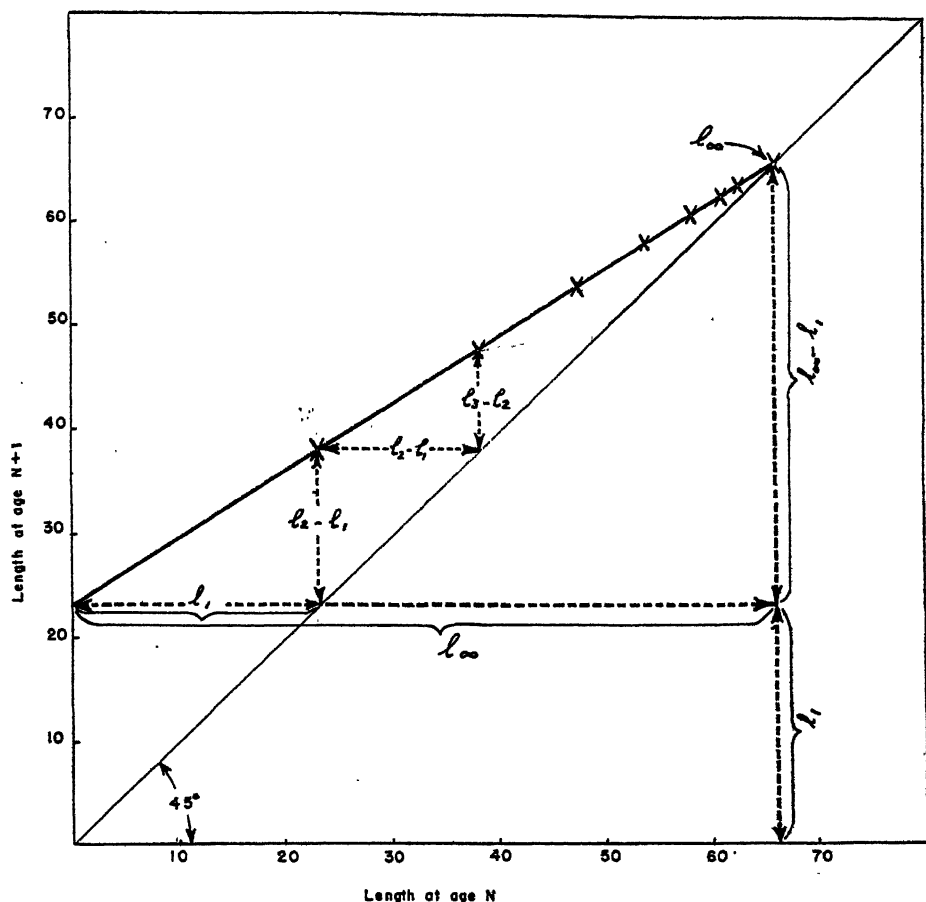


FIGURE 4. Transformation of the growth curve of a hypothetical animal, drawn in a heavy line to illustrate the dimensions given in the text. Note that the ultimate length, l_{∞} , can be located graphically as the point where the length at age n equals the length at age $n+1$; also where the transformation intersects a line drawn at 45° through the zero point.

Therefore,

$$l_n = l_1 + k l_1 + k^2 l_1 + k^3 l_1 + \cdots + k^{n-1} l_1 = l_1 \frac{1 - k^n}{1 - k} \quad (4)$$

by the well-known formula for the geometric series. Therefore, when n approaches ∞ , l_n tends to l_{∞} , and k^n to 0, and so we obtain

$$l_{\infty} = \frac{l_1}{1 - k} \quad (5)^3$$

³ Where this graphical representation gives precisely a straight line, the above calculation shows that l_n is expressed by the formula $l_n = l_{\infty}(1 - k^n)$. It is a modification of the "Modified Exponential" (cf., Croxton and Cowden, 1940, pp. 441 ff.), but contains one less parameter, and of course applies only to that segment of the growth curve above the inflection point.

Thus the limiting length l_{∞} may be computed readily from the y intercept, l_1 and the slope of the fitted straight line, k ; and k , in turn, is readily calculated from any of the ratios preceding equation (1).

According to the series of equations (1) to (4) the slope of the transformed growth curve, k , is the constant given not only by the ratio $\frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$ but also by the ratio $\frac{l_{\infty} - l_{n+1}}{l_{\infty} - l_n} = k$.

In other words, the amount of growth which remains unfulfilled at the beginning of any time interval, is a constant percentage of the amount of it which had remained at the beginning of the preceding time interval. Consequently the higher the k value, the more slowly growth approaches the limiting length.

This method of plotting growth data permits an objective determination of the limiting length, l_{∞} , even before that length is "attained." ⁴ It provides two growth characteristics, k and l_{∞} , from which the upper segment of the length-on-time growth curve can be reproduced. These constants are so simply derived, that it is practical to determine them for large numbers of individuals. They can be used for studying growth variation within and between populations, hence for distinguishing between races of animals with differing growth patterns. The method is particularly useful in studies of fishes whose scales bear annual rings, from which the growth history of individual specimens can be estimated.

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⁴Strictly speaking, of course, under the terms of this description the limiting length is only approached; it is never attained.

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INVESTIGATION ON THE LOCUS OF ACTION OF DDT IN FLIES (DROSOPHILA)

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INTRODUCTION

The experiments reported herein were designed to gain information as to where DDT produces its poisonous effect in the insect.

DDT poisoning in insects is characterized by symptoms of hyperactivity and discoordination of neuromuscular system, followed by convulsions and terminating in death. Isolated legs of DDT treated roaches continue to twitch after complete separation from the body, and isolated legs of normal roaches were induced to twitch by the application of DDT to the cut surface, although they remained quiescent if DDT was not applied (Yeager and Munson, 1945). Likewise the isolated legs of adult blowflies (*Phormia regina*) showed twitching movements when dipped before or after they were cut off from the animal into a one per cent DDT acetone solution, while untreated isolated legs remained motionless (Chadwick, 1945). From somewhat different experiments, Tobias *et al.* (1945) working with roaches suggested that the thoracic ganglia were the critical loci for the action of DDT. From these observations it appeared likely that the symptoms of DDT poisoning in insects resulted from an effect on the central nervous system, but that there existed also peripheral components which were not as yet exactly delimited.

MATERIAL AND METHODS

The experiments were performed on the larvae and adults of the fruit-fly (*Drosophila virilis*). The DDT preparation used was in form of an emulsion, (one per cent DDT, one per cent lecithin, ten per cent peanut oil, emulsified in a 0.95 per cent NaCl solution). This emulsion was injected by means of a micro-pipet into the abdominal cavity of the insect. The physiological saline solution used throughout the investigation was a Ringer solution modified for *Drosophila* (H_2O , 1000 cc.; NaCl, 7.5 gm.; KCl, 0.35 gm.; $CaCl_2$, 0.21 gm.). The various concentrations of phenobarbital used were also always made up in this Ringer solution. Imaginal discs were transplanted with the usual *Drosophila* transplantation technique.

EXPERIMENTAL

Behavior of larvae and adults after poisoning

When DDT emulsion was injected into the abdomen of adult flies which had been slightly narcotized with ether, the response to the poison was immediate. Legs and wings at once went into violent, uncoordinated movements. About twenty seconds after the injection the abdomen, previously motionless, began to

convulse; its movements were a rapid succession of short, uncoordinated spasmodic twitches. At first the convulsions were strong and a great deal of the injected emulsion was thus pressed out through the puncture wound. About five minutes after the injection the legs and wings went into a spasm and took up a characteristic position. The legs were drawn toward the body and crossed over ventrally, while the wings were folded backward. This position was maintained until the animal died. The contractions of the abdomen continued for about four to seven hours but became gradually weaker. After seven hours the animal was apparently dead. Although during all of this time the legs and wings remained in their spasmodic condition, one occasionally observed slight twitches of the tarsal segments and of the antennae; the wings, however, showed no movement. It was thus clear that the muscular response to the poison varied in different regions of the body, for the wing and leg muscles soon went into contraction and remained that way, while the muscles of the abdominal wall continued to convulse for a long period.

When an emulsion prepared in the same manner, but containing no DDT, was injected into flies, no effect was noted. The animals recovered from narcosis in the usual way, and were still alive the following day without any apparent injurious effects. Thus the symptoms described above were due to DDT and were not caused by the emulsion itself.

Larvae narcotized with ether were motionless, except for the pulsating heart tube visible through the transparent skin. When such larvae were injected with DDT emulsion, one observed at first a great acceleration of the heart-beat. Convulsions of the body wall began about twenty seconds after the injection. It was difficult to observe the heart-beat while the convulsions were in progress, but it was found in ligatured larvae, which will be described below, that the heart-beat soon became normal again after the initial acceleration. The larval convulsions were very strong and uncoordinated. Contraction wave after contraction wave passed over the creature, somewhat resembling crawling movements, yet the animal was unable to move from its place. The forward and backward movements were much more rapid than the normal crawling movements. Moreover, the animal never extended to its full length but remained partly contracted all the time. Short twitching contractions occurred in various parts of the body, and broke the wave-like contraction into a complex, uncoordinated movement. The larvae moved continuously in this way, some of them for twenty or more hours. The majority of such larvae died within ten hours, but some of them lived for twenty-three hours after the injection. The symptoms were the same, whether last (3rd) or late 2nd instar larvae were used for the experiments.

In control experiments, where emulsion containing no DDT was injected, no such symptoms occurred.

ETHER NARCOSIS AND DDT SYMPTOMS

Normal flies etherized only until their movements stopped were completely relaxed if removed at that time from the ether. Their wings were in normal resting position and their legs were bent in the way assumed by flies at rest. Such flies recovered about one half hour after their removal from the anaesthesia and then seemingly behaved normally. Yet flies left for a longer time in the etherizer behaved quite differently. Their wings were folded back and their legs were

stretched out and held stiffly away from the body. Usually flies thus over-etherized did not recover from the narcosis and died in that position.

The reaction of these differently etherized flies to DDT poisoning was quite interesting. The slightly etherized individuals showed, after DDT injection, the typical DDT symptoms described in the foregoing section. On the other hand, when over-narcotized flies were injected with DDT, one noticed that their legs and

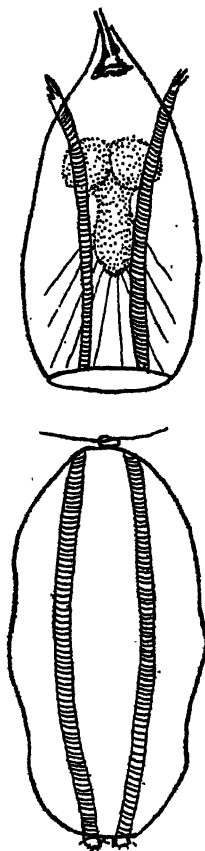


FIGURE 1. Diagram illustrating larval ligature experiments for separating the central nervous system from the rear part of the body. Note location of central nervous system (stippled) in anterior part.

wings did not respond to the poison. Since such flies never recovered from the narcosis it might be thought that they were already dead at the time of the DDT injection. This, however, was not the case, for their abdomens showed the typical DDT convulsion. These convulsions continued for about three hours, but were somewhat weaker than those of the slightly etherized animals. About that time the uninjected but over-etherized control animals were still completely immobile and apparently dead. In comparing the uninjected and injected flies, one gained the impression that the DDT treatment in some way partly released the ether block.

THE IMPORTANCE OF THE CENTRAL NERVOUS SYSTEM

The central nervous system of *Drosophila* is concentrated in the anterior part of the body. In the larva the central nervous system is located in the third thoracic and the first abdominal segments; it consists of the two brain hemispheres, to which is attached a large ganglionic mass (Figs. 1 and 3). This large ganglion is a compound structure, for it includes the sub-oesophageal ganglion, the three thoracic ganglia, and the eight abdominal ganglia. In the adult insect the three thoracic ganglia are separated, but all eight abdominal ganglia unite into one ganglionic mass which extends into the first segment of the abdomen (Fig. 2).

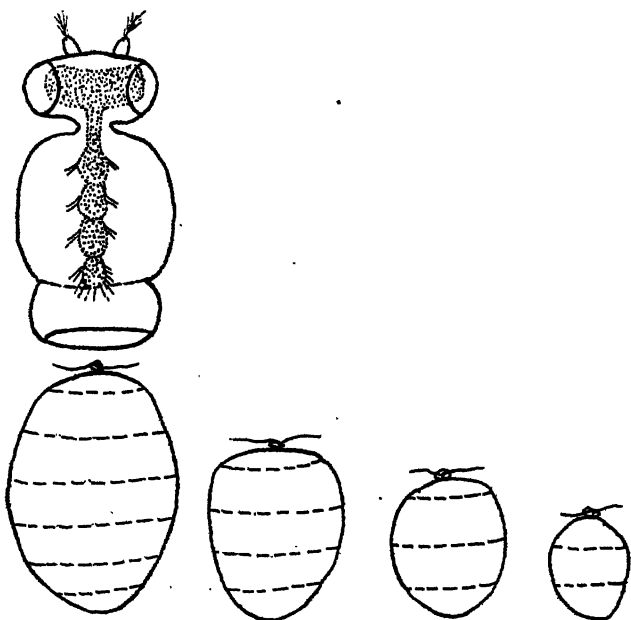


FIGURE 2. Diagram illustrating ligature experiments on adult flies, showing the separation of progressively smaller abdominal parts from the anterior region of the animal. Note location of central nervous system in anterior part of fly.

The described topography allowed one to separate experimentally large parts of the insect body from its central nervous system. The separation was accomplished by means of a fine silk ligature tied around the body of the animal. Depending upon the position of the ligature, smaller or larger parts of larvae and adults were thus isolated. In all experiments of this kind, the part in front of the ligature was cut away, in order to be sure that no connection to the ganglia remained (Figs. 1 and 2). Abdominal parts isolated in this way were completely motionless and stayed alive for several days.

The most useful symptom for the experimental approach was the contraction of the abdominal wall muscles under the influence of DDT. This effect was very uniform, clearly observable, and lasted a considerable time. The question then arose: were these movements under the control of the central nervous system?

In other words, was the muscular activity caused by an effect of DDT on the central nervous system? To test this possibility, isolated portions of larval and adult abdomens containing no ganglia were injected with DDT emulsion. The response to the injection was called forth immediately. Both larval as well as adult abdomens exhibited the same typical movements that were observed in the abdomens of injected intact flies. Again, as in the intact animals, it was found that the isolated adult abdomen responded somewhat faster than the isolated larval abdomen. That is, the larval abdomen responded at about thirty-sixty seconds and the adult abdomen about twenty seconds after the injection. The DDT convulsions of the isolated parts continued for a considerable time, but not as long as in the intact animals. In the isolated adult abdomen the convulsions clearly became weaker two hours after injection, yet weak contractions were observed five hours after the injection. In the isolated larval abdomens the contractions were still strong four hours after the injection, yet these parts never survived for twelve hours as injected whole larvae commonly did.

A similar sequence of events was observed when only the distal two or three segments of the adult abdomen were isolated from the rest of the body and then injected with DDT emulsion (Fig. 2) or when only two or three segments from the middle of the larval abdomen were isolated by means of two ligatures and then injected.

These findings showed that the central nervous system did not control the abdominal symptoms produced by DDT, since they occurred also in the absence of the central nervous system. However, it must be recognized that this does not imply that the central nervous system was unaffected by DDT.

BODY FRAGMENTS AND DDT SYMPTOMS

In order to reduce the structural complexity of the insect body, in an effort to localize more closely the site of action of DDT, the following fragmentation experiments were performed. Rectangular pieces of skin were cut from the dorsal, lateral, or ventral wall of adult flies and placed in a drop of saline solution. These pieces, about two segments wide, included some muscles of the body wall, fat tissue, tracheae, and nerves but no ganglia. The pieces of dorsal body wall included also part of the heart tube and some alary muscles. In saline solution these pieces remained motionless, but if a few drops of DDT emulsion were added, the isolated muscles in the piece began to twitch. This response began about thirty seconds after DDT was added and continued for about two or three minutes. In control experiments where emulsion containing no DDT was added, no such response occurred.

These experiments confirmed those above by showing that the central nervous system was not necessary for the response of the abdominal muscles to DDT. It would appear that DDT affected either the muscles directly or the peripheral nerves. One way of distinguishing between these two possibilities would be to test a nerve-free muscle preparation with DDT. Technically, however, it is impossible to obtain such a preparation. Other methods were sought to settle this question and are described below.

TREATMENT WITH PHENOBARBITAL

Phenobarbital is a drug which depresses central nervous activity in vertebrates. The effect of this substance on flies has not been described previously. When a ten per cent phenobarbital saline solution was injected into the abdomen of adult flies the animals were apparently killed instantly. The slight vibrating movements, characteristic of lightly etherized flies stopped immediately after the injection. All of the muscles seemed to relax and the legs and wings were held in normal position.

Flies which had just come out of ether narcosis but were still sluggish lost their coordination when injected with a one per cent phenobarbital solution. Wings, legs, and abdomen moved uncoordinatedly for several hours until the animal finally died. The movement of the abdomen, also uncoordinated, was very different from the symptoms produced by DDT.

Injection of 0.1 per cent phenobarbital solution into adult flies induced narcosis, followed by complete recovery of the animal. One hour after the injection some flies crawled about slowly, while others were still unable to hold themselves up and fell over from time to time. By this time the effect of the ether had worn off and these symptoms were regarded as phenobarbital effects. One-half hour later the coordination of the animals had improved but their movements were still slow. But the next day the animals had recovered and behaved normally.

That phenobarbital affects the nerves rather than the muscles in flies was indicated by the following experiments. Fully grown larvae were split open along the mid-dorsal line. The intestine, Malpighian tubes, fat body and the main tracheae were then removed. Care was taken not to disturb the brain and ganglia in the anterior part of the body. This manipulation was carried out in physiological salt solution. The skin with its muscular layer and the adhering nervous system was placed in a small dish with a wax bottom and covered with fresh physiological salt solution. The preparation was then stretched out and by means of fine pins was fastened to the wax bottom of the culture dish, as shown in Figure 3. In this condition the tissues stayed alive for several hours. From time to time the muscles contracted, showing the typical wave-like contraction pattern of a crawling larva. After these movements had been observed for ten minutes the physiological solution was removed and replaced by a one per cent phenobarbital solution. The movements in the preparation stopped immediately. Again, after about ten minutes during which time no movements occurred the phenobarbital solution was removed and replaced by physiological saline. This solution was in quick succession drawn off and replaced about two or three times. The preparation was thus washed clean of any phenobarbital. It was now observed that normal movements had returned.

Ten minutes later the physiological solution was removed and replaced by one per cent phenobarbital solution; all movements again stopped. When washed and placed in physiological solution again the movements in the preparation reappeared. This procedure was repeated three or four times at intervals of about ten minutes and still the tissues continued to contract spontaneously when in physiological solution.

These experiments indicate that the one per cent phenobarbital solution was apparently not injurious to the tissues for the length of exposure tested. Moreover, while in phenobarbital, no muscular movement was observed, yet if the

muscles were stimulated directly by touching them with a fine needle, localized contraction in the region of the stimulus was noted. Following the mechanical stimulus the activated muscles contracted rather rapidly, stayed in the contracted state for some time and relaxed very slowly. This localized response of the muscles to mechanical stimuli when under phenobarbital indicates that the drug had not paralyzed the muscles directly but rather the nerves.

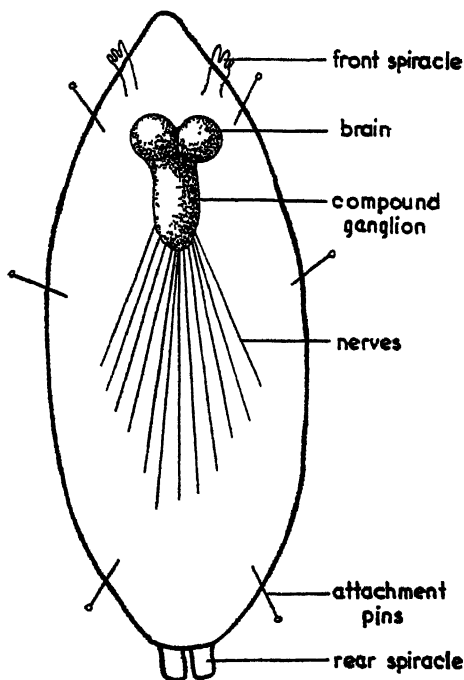


FIGURE 3. Diagrammatic representation of the skin muscle preparation of a whole larva, pinned to the wax bottom of the culture dish.

COMBINED PHENOBARBITAL AND DDT TREATMENT

The knowledge that phenobarbital apparently acted on the nerves but not on the muscles provided a tool for determining whether DDT affects the nervous tissue or the muscles. If DDT could affect the muscles directly DDT symptoms should be provoked in animals paralyzed by phenobarbital. If, on the other hand, DDT affects only the nerves, no DDT symptoms should occur in animals treated with phenobarbital. That the latter is the case was shown by the following experiments.

In physiological solution the muscle preparation (Fig. 3) showed spontaneous crawling movements as described above. If a small amount of DDT emulsion was dropped onto such a preparation, the rhythm of the movements was interrupted; it became uncoordinated rapidly and resembled the movements observed in DDT-treated larval abdomens. If, on the other hand, DDT emulsion was dropped in the same manner onto preparations kept in one per cent phenobarbital solution, no

movement whatsoever took place. Moreover, if the physiological solution containing DDT emulsion was washed off from the actively moving preparation, and was replaced by one per cent phenobarbital solution, the movements ceased immediately. However, it was impossible to bring the preparation once treated with DDT back to its normal way of movement by washing the DDT solution off and replacing it with pure physiological solution. Such preparations still showed DDT symptoms. Apparently it was impossible to wash all the DDT out by the methods used. The DDT symptoms were of course stopped by placing the preparation again into phenobarbital.

Similar results were obtained when DDT-treated larval or adult abdominal parts were injected with one per cent phenobarbital solution. The DDT symptoms of such parts ceased immediately after phenobarbital was administered by injection. The reciprocal experiment, where phenobarbital was injected first, yielded the same results, for when DDT was injected into such treated abdomens no DDT symptoms occurred. The symptoms of whole larvae or adults which had been treated with DDT were also stopped immediately by injecting a one per cent phenobarbital solution.

Certainly the effects of phenobarbital on isolated, DDT-treated abdomens indicate that the absence of the central nervous system did not limit the action of the drug, showing that in insects phenobarbital may act on the peripheral nerves.

At this point the discussion of the phenobarbital effects on DDT poisoning must be augmented by an experiment showing how phenobarbital in weaker concentrations caused at least a partial recovery from DDT poisoning. It has been stated before that the legs and wings of adult flies injected with DDT were completely paralyzed five minutes after the injection. Now, when 0.1 per cent phenobarbital solution was injected into such animals, the movements of their legs were restored. These movements were uncoordinated and were similar to those observed in the beginning stages of DDT poisoning before the organism went into spasm. Also, the wings were able to move somewhat and were not folded backward. Even the convulsions of the abdomen were much less pronounced. Leg movements continued for about two hours, which of course was a much longer time than ever noted in animals injected with DDT only. These findings clearly show the antagonistic effect of phenobarbital on DDT.

CAPACITY OF DIFFERENT TISSUES FOR GROWTH AND DIFFERENTIATION AFTER DDT POISONING

If it is true, as the experiments seemed to indicate, that DDT affects only the nervous system, one might expect other tissues to be largely unharmed by the DDT treatment. This expectation can be tested experimentally. It is known (Bodenstein, 1943) that larval tissues will grow and differentiate normally when transplanted into the abdomen of larval or adult flies. In the larvae the transplanted tissues will develop in synchrony with their hosts to imaginal completion, and in adult flies, the transplant will undergo a considerable amount of growth. The capacity of the tissue for growth and differentiation, it is believed, offers a good criterion for testing the condition the tissue is in after being exposed to the poison. Tissues affected by DDT should certainly not develop normally. The following experiments were designed to clarify this issue.

Fifteen last-instar larvae were injected with DDT and placed on moist filter paper. Twenty-two hours later four larvae were still alive and showed typical DDT symptoms. Three of these larvae were opened and their eye discs, leg discs, and antennal discs dissected out. These discs are the primordia for the future eye, leg, and antenna of the adult fly.

They were transplanted into the abdomen of the mature larvae, one disc to each host. The several hosts carrying the transplants from one donor were kept separated from the hosts carrying the transplants of the other donors.

If the transplanted organs were able to develop normally, they should have been found as fully differentiated organs in the abdominal cavity of their respective hosts after metamorphosis. From the fifteen original hosts comprising the cases of all three series, eleven survived the operation. These animals completed their metamorphosis seven days after the operation and emerged. They were then dissected. Three completely differentiated legs, one eye, and one antenna supplied by the first donor were recovered. Hosts which received transplants from the second donor yielded three legs, two antennae, and two eyes, and from the third donor, three legs. All transplants were fully differentiated. As far as the detailed morphological differentiation of the tested organs was concerned, they were found to be completely normal, and there was no reason to believe that the histological differentiation likewise was not normal.

For another experimental series, five leg discs, dissected from the fourth living larva of the original set of DDT-treated animals, were transplanted into the abdomen of five adult flies. Each host in addition to the leg disc also received two ring glands. This structure is necessary for the continued growth of the transplant, for it furnishes a growth-promoting hormone (Bodenstein, 1943). Three days after the operation one host was killed and the leg disc dissected. It had grown but little. Three other hosts were killed six days after the operation and the transplants dissected. These leg discs had clearly become larger. Finally the last host was killed 24 days after the operation and the disc dissected. In this case the transplant had grown considerably and had reached an advanced state of differentiation. The results of this series of experiments were very similar to those obtained in transplanting normal discs in the same manner (Bodenstein, 1939 and 1943).

In conclusion, these two experimental series show that exposure to DDT for twenty hours in no way affected the capacity of the imaginal tissues for growth and differentiation. Hence these findings are further evidence that the nervous system alone is affected by DDT.

SUMMARY

1. The larvae and adults of *Drosophila virilis* were fatally poisoned by injecting a one per cent DDT emulsion into the abdominal cavity. The poison produced a typical pattern of symptoms.

2. The neuromuscular system of the wings and legs was apparently very sensitive to the poison, for they went into spasm long before the muscles of the abdominal wall. There was also a difference in sensitivity to the poison between the larva and adult, the larva being more resistant to the DDT emulsion.

3. Phenobarbital was found to affect the nervous system. Paralysis by phenobarbital was also produced in the absence of the central ganglia. This shows that the drug also affected the peripheral nerves. Muscles of larvae treated with phenobarbital responded to mechanical stimulation.

4. Since DDT produced no symptoms in animals treated with phenobarbital and since animals treated with DDT lost their DDT symptoms when injected with phenobarbital, it was shown that DDT acted on the nervous system. Moreover, body parts which had been isolated from the central nervous system and then treated with DDT stopped convulsing after phenobarbital administration. This shows that DDT affected the peripheral nerves.

5. The methods used do not allow one to determine what part of the peripheral nervous system might be affected. There are three possibilities. The poison might affect (1) the motor nerves leading to the periphery; (2) the myoneural junctions; (3) the peripheral nerve net. It is however still questionable whether such a nerve net exists in *Drosophila*.

6. The antagonistic effect of phenobarbital on DDT was clearly indicated by the fact that the spasm of the legs and wings in DDT-treated flies was partly relieved by treatment with phenobarbital.

7. The conception that only the nervous system is affected by DDT has been strengthened by the fact that larval organs (imaginal discs) which had been exposed to DDT for twenty hours grew and differentiated normally when transplanted into untreated larvae.

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THE CONTRACTILE VACUOLE AND THE ADJUSTMENT TO CHANGING CONCENTRATION IN FRESH WATER AMOEBAE

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INTRODUCTION

In considering the relationship existing between an organism and its environment, it is necessary to distinguish clearly between the environment of the organism and the environment of the individual cells. The effect of the environment on an organism will depend specifically on the extent to which the cellular environment is controlled by the organism as a whole. As pointed out by Krogh (1939), the osmotic environment of the tissue cells of the vertebrates—the body fluid—is maintained at a very constant level. This is true to a lesser degree among the lower organisms. In the free-living protozoa, the cellular environment is the outside environment of the organism and is controlled little, if at all, by the organism. We find consequently that the individual cells of higher organisms have lost their resistance to osmotic changes while the cells of many of the protozoa, of necessity, have retained the ability to withstand considerable variation in the osmotic environment. It is well known that certain bacteria and molds can withstand enormous osmotic changes—which also have been shown to be true of a considerable number of protozoa (Hopkins, 1938; Butts, 1935; Mast and Hopkins, 1941; and Kitching, 1938).

The nature of the adjustments made by cells capable of withstanding great osmotic changes is not clearly understood. In the literature, the assumption is rather generally made that the osmotic concentration of the cellular contents must be maintained at a fairly constant value in order to be consistent with the metabolic processes. This assumption is made, regardless of the fact that evidence for the constancy of the intracellular osmotic pressure is conspicuously absent, especially for the lower organisms. We find that the prevailing opinion as to the function of the contractile vacuole of the protozoa is that it is an organelle which operates to maintain the intracellular osmotic pressure at a relatively constant level which is higher than that of the environment. This theory maintains that excess water, diffusing through the outer membrane into the hypertonic cell, is extracted, isolated into the vacuole, and thus is eliminated when the vacuole is discharged (Kitching, 1938). However, Mast and Hopkins (1941) have shown that the marine *Amoeba mira* (*Flabellula mira* Schaeffer), which can withstand enormous changes in the

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concentrations of the salts of sea water, does not maintain a constant intracellular osmotic pressure. The intracellular pressure varies with that of the environment. They have shown that, if the amoeba is living in, and is adjusted to a medium, the intracellular pressure is always only slightly higher than that of the environment. Thus, a change in the osmotic pressure of the environment results in a corresponding change in the intracellular pressure.

In view of the fact that *Amoeba mira* is a marine organism and does not form contractile vacuoles even in dilute media, it was considered important to extend the investigations to a fresh water amoeba, which forms and eliminates contractile vacuoles, in order to ascertain whether these forms actually maintain a constant osmotic pressure by means of the action of the contractile vacuoles. The amoeba selected was a fresh water form collected from a swamp near Chicago. A single amoeba was isolated and allowed to multiply on an agar culture, using the methods of Rice (1935). All experiments were performed on the progeny of this amoeba.

METHODS AND RESULTS

Description of the amoeba

As in the case of most amoebae, it is difficult to decide upon its correct name. Figure 1 is a composite diagram illustrating its structure as observed in fresh water. Dujardin's *Amoeba lacerata* (1841) fits it fairly well. We shall then adopt the name of *Amoeba lacerata* Dujardin. Schaeffer (1926) has placed amoebae of this type in the genus *Mayorella* Schaeffer. Thus, using the system of Schaeffer, the name is *Mayorella lacerata* Dujardin.

Amoeba lacerata is a small amoeba, rounds up readily when disturbed, and in that condition has an average diameter of fifteen micra or a volume of about 1500 cubic micra. It varies from a limacine to a flattened fan shape and has a clear hyaline border (Fig. 1a) which is thick anteriorly and very thin posteriorly. A few, fine, pointed, indeterminate pseudopodia may project from the surface—anteriorly, superiorly, and posteriorly. Cytoplasmic granules are numerous—(1) an abundance of small, barely visible granules and (2) larger granules (or vacuoles) which are less numerous and more variable in number and size (Fig. 1b). The living nucleus (Fig. 1d) is spherical with a central endosome. Food is engulfed into the under surface as the amoeba moves over the food particles. Except in rare cases, food is engulfed with little or no water. Ingested food particles coalesce with clear vacuoles (Fig. 1c) of the cytoplasm, and the resulting small food vacuoles coalesce with each other as they do in *Amoeba mira* (Hopkins, 1938) to form large food vacuoles (Fig. 1f). These vacuoles are readily stained with Nile blue sulfate and neutral red. Egestion of food vacuoles (Fig. 1g) occurs at the posterior tip.

No direct connection or relationship between the food vacuoles and the contractile vacuoles has been observed except that occasionally a food vacuole may be ruptured into the contractile vacuole instead of to the outside. At egestion, the food vacuoles are rarely large and watery. Just before egestion they may be observed to decrease slowly in size until little more than food residues remain. This would indicate a protoplasmic absorption of water from the food vacuoles. It is also interesting to note that at the time when this apparent absorption takes place, the food vacuoles are located posteriorly and in close proximity to the region of

contractile vacuole formation which MacLennan (1941) and others have called the nephridioplasm. It is entirely possible that the water leaving the food vacuoles is absorbed into the contractile vacuoles.

The contractile vacuoles

In an amoeba moving forward rapidly, the contractile vacuoles form at the posterior end, as shown in Figure 1c. When the individuals are flattened out and feeding actively, it is difficult to distinguish the posterior end unless the location

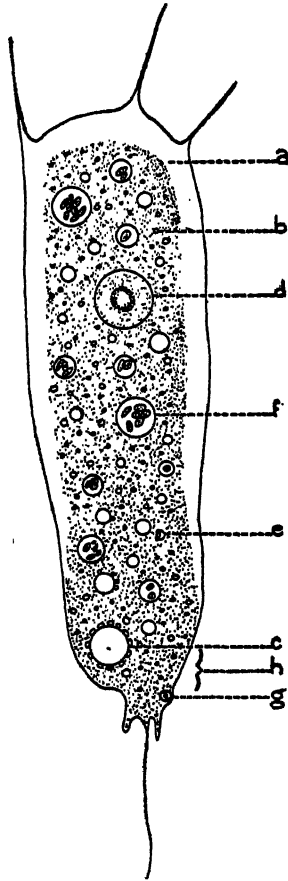


FIGURE 1. Diagram showing the characteristic structure of *Amoeba lacerata* Dujardin. *a*, hyaline border; *b*, large cytoplasmic granule (vacuole); *c*, contractile vacuole; *d*, nucleus; *e*, small clear vacuole adhering to a recently engulfed bacterium; *f*, food vacuole; *g*, food vacuole in position for egestion; *h*, nephridioplasm.

of the contractile vacuoles is accepted as a distinguishing feature of the posterior part of the cell. Observations on the origin of contractile vacuoles in active amoebae have revealed the following interesting facts:

(1) They develop in the posterior third of the cell, or, if they begin to develop in an apparently anterior part of the cell, that part will soon become the posterior part—as evidenced by change in the direction of protoplasmic streaming. If the posterior region of the amoeba becomes separated into two parts by a large food vacuole, contractile vacuoles will arise and be expelled independently on both sides. Pressure on the amoeba by a coverslip will cause contractile vacuoles to arise and be expelled independently in several parts of the cell. This phenomenon was also observed by Howland (1924b) in *Amoeba verrucosa*. A freely moving amoeba, as a rule, has only one formative region for contractile vacuoles.

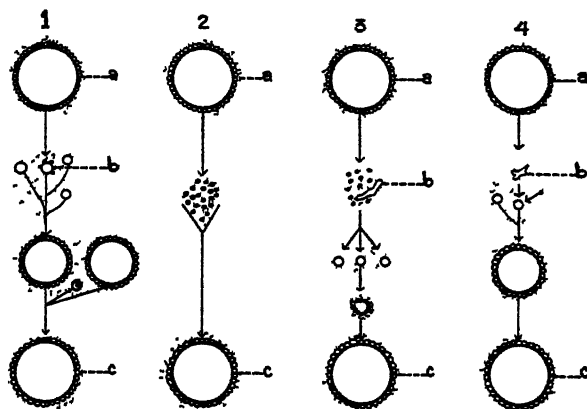


FIGURE 2. Diagrams showing contractile vacuole cycles, 1, 2, 3 and 4. Vacuoles *a*, are expelled to outside leaving residues, *b*, which by coalescence with other vacuoles, small and large, and swelling, form vacuoles, *c*, which are expelled

(2) The nephridioplasm (Fig. 1*h*) is in the region in which plasmogel is rapidly becoming plasmosol. This region is often under considerable pressure (Mast, 1926). This is evidenced in *Amoeba lacerata* by the observations that the contractile vacuoles are often squeezed into oblong shapes, that food vacuoles and contractile vacuoles which normally never coalesce are sometimes squeezed so tightly together that the food vacuoles rupture violently into the contractile vacuole. The contraction of the contractile vacuole is, however, not always violent. Sometimes the contraction is very slow, while at other times, it may be only partial.

(3) While the contractile vacuoles increase in size by absorption, as will be shown later, the most conspicuous methods of growth are the coalescence of small droplets or vacuoles with one another, of large vacuoles with each other, and of small vacuoles with large vacuoles (Fig. 2). Small vacuoles less than 0.5 micron in diameter—can be observed coalescing with large vacuoles whose diameters are many times greater. Careful focusing on the outer boundary of a large contractile vacuole reveals this phenomenon. The coalescence of two contractile vacuoles is seldom violent. It occurs at about the same speed as the coalescence of two drops of olive oil.

(4) When a vacuole contracts, its contents are expelled to the outside. This occurs usually when the vacuole has a diameter of about 4 micra, but often smaller vacuoles contract, and larger ones may be formed before contraction occurs. Rup-

ture usually takes place through the postero-superior surface of the cell. Sometimes the contracting vacuole leaves no residue, but usually a small residue (Fig. 2b) remains, which coalesces with other small vacuoles forming new, large vacuoles. The residues are usually spherical, but often may assume oblong, stellate, or other shapes. Whether a residue remains or not, the region from which the previous vacuole was expelled contains small vacuoles which coalesce to form a large vacuole (Fig. 2c). These small droplets, or vacuoles, are continually appearing in the nephridioplasm. The region is generally devoid of food vacuoles.

The diagrams in Figure 2 illustrate four sequences which have been observed to occur in the nephridioplasm.

(5) All efforts to associate the contractile vacuoles with cytoplasmic granules of any sort have been futile.²

Tolerance of Amoeba lacerata for changes in the concentration of the medium

The swamp water from which the amoeba was collected was very dilute. The amoebae were first cultured in water from Lake Michigan to which grains of wheat were added. Attempts to culture it in various dilutions of sea water proved quite successful. It can be transferred, without deleterious effects, directly from lake water cultures to 5 per cent sea water to which grains of wheat are added. Excellent cultures of healthy amoebae develop within five days. Direct transference of the amoebae from 5 per cent sea water cultures to similar cultures made up in various dilutions of sea water results in excellent growth, even when the percentage is as high as 50 per cent. Direct transference to cultures having percentages higher than 50 per cent results for the most part in complete failure or very slow growth. If, however, the amoebae are first cultured in 50 per cent sea water and then transferred to 75 per cent or 100 per cent, survival and growth result. Slow growth may even be obtained in 125 per cent by adjusting the amoebae to 100 per cent and then transferring them to 125 per cent sea water.

The volume of the amoebae and the concentration of the culture medium

A study of the relation between the volume of the amoebae and the concentration of the culture medium in which they were grown has been made. The amoebae for this study were all from the same 5 per cent sea water culture and were inoculated into cultures of varying concentrations from one per cent to 75 per cent sea water. To each culture five sterile grains of wheat were added. Five cultures were set up at each concentration in exactly the same way and at the same time. When abundant growth and reproduction had occurred in all the cultures the amoebae were caused to round up by pipetting the culture fluid back and forth. Their diameters were then measured under the microscope with an eyepiece micrometer. At least 100 amoebae were measured for each concentration. The age of the cultures was the same at the time of measurement. A total of over 1,750 amoebae were measured. The results are presented in Figure 3, in which the average volume is plotted against the concentration of the culture. The increase in volume with an increase in concentration appears significant statistically. (The standard errors are graph-

² The very small vacuoles which form the larger contractile vacuoles undoubtedly have been mistaken for granules by various authors,

ically indicated in the curve by the length of the vertical lines drawn through the points.) The total range of variation in average volume is, however, only a little over $750 \mu^3$. This increase in volume with concentration, if significant, is probably due to a lower rate of fission in respect to growth in higher concentrations than in lower concentrations. It should be mentioned here, however, that much greater

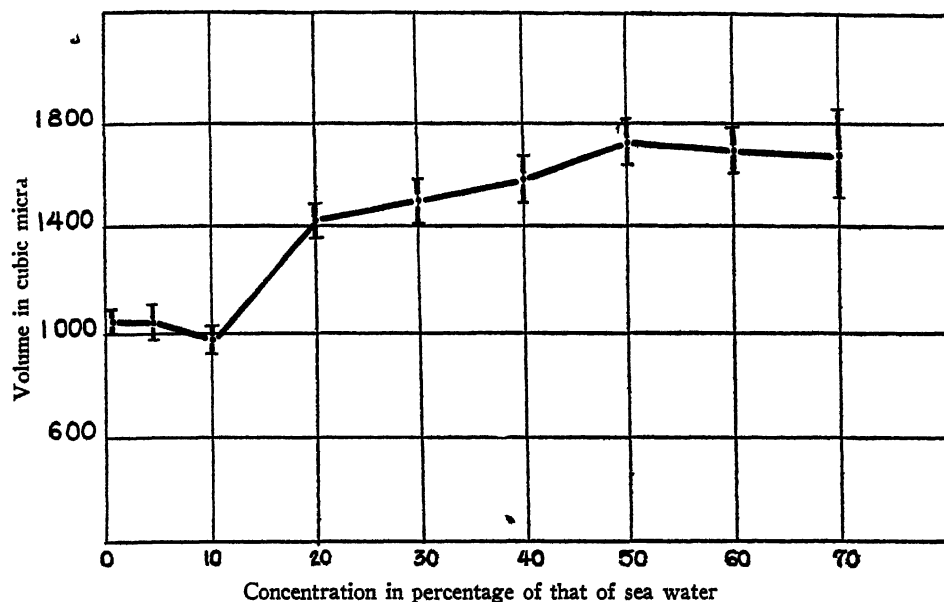


FIGURE 3. Graph showing relation between volume of amoebae and the concentration in which they are grown.

variations occur in average volume of amoebae from cultures when the nature of food is allowed to vary more than was the case in this series of cultures. (See average volumes of cultures of Figure 4.)

Relation between the protoplasmic osmotic pressure and osmotic pressure of the external medium

In amoebae which have become adjusted to 50 per cent sea water, is the cytoplasmic concentration the same, higher, or lower than that of amoebae adjusted to 5 per cent or 20 per cent sea water? What relation does the cytoplasmic concentration bear to the concentration of the culture medium?

Since these amoebae round up into spheres when agitated, the osmotic concentration of the protoplasm can be readily approximated. When the amoebae are placed in sea water of a given strength with a resulting decrease in volume, we can safely conclude that the osmotic pressure is higher externally than internally. If, on the other hand, the volume of the amoebae increases, the osmotic pressure of the protoplasm is greater than that of the medium. If, however, the plasmalemma is permeable to the solutes of the medium the permanence of the change will depend upon the degree and speed of solute penetration.

The results of experiments designed to answer the above questions were as follows:

Amoebae raised in 5 per cent sea water, and consequently adjusted to it, are placed in one per cent sea water. They immediately swell to a maximum volume and then gradually decrease in volume until approximately their original volume is regained. The smaller the change in concentration, the more rapid is the return to original volume. The same is true when amoebae cultured in any strength in which they will live are placed in higher and lower concentrations.

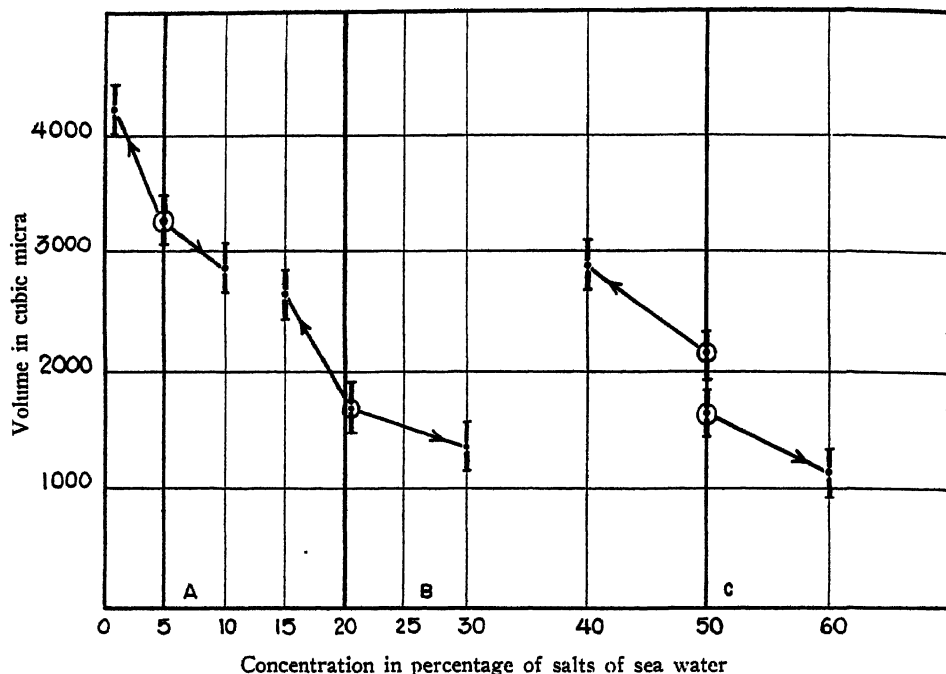


FIGURE 4. Graph showing volume change occurring immediately when amoebae grown in various percentages of sea water are transferred to lower and higher percentages respectively. A, amoebae grown in 5 per cent transferred to one per cent and 10 per cent; B, grown in 20 per cent and transferred to 15 per cent and 30 per cent; C, grown in 50 per cent and transferred to 40 per cent and 60 per cent.

The results of a statistical study of the initial changes in volume when the concentration of the medium is increased or decreased from that of the culture medium are given in Figure 4. In this experiment the amoebae were cultured in 5 per cent, 20 per cent, and 50 per cent sea water (Fig. 4A, B, and C respectively). The amoebae were agitated in the culture; the diameters of from fifty to one hundred individuals measured; the volumes calculated and averaged. The concentration was increased on some and decreased on others; and then the volumes of fifty amoebae whose medium was concentrated, and fifty amoebae whose medium was diluted were measured and averaged. Measurements of volume were all made between 5 and 30 minutes after the concentration was changed. In the figure, the

circles give the average volume of the amoebae in culture medium. The other points represent the average volume of amoebae which have been transferred to the concentration indicated. Volume is indicated on the ordinates. The concentration of the culture is indicated on the abscissas. The length of the vertical lines through the points represents the extent of the standard error.

These results indicate very definitely that the osmotic pressure of the protoplasm of *Amoeba lueerata* varies in the same direction as that of the external medium as was found to be the case in *Amoeba mira* (Mast and Hopkins, 1941). The osmotic pressure of protoplasm of amoebae cultured in 5 per cent is not over that of 10 per cent, nor under that of one per cent sea water. The osmotic pressure of those cultured in 20 per cent sea water is not over that of 30 per cent nor under that of 15 per cent. Finally the osmotic pressure of those cultured in 50 per cent sea water is not over that of 60 per cent nor under that of 40 per cent. Due to the rapid return of the amoebae to their original volume when small concentration changes were made, we were unable to make more accurate measurements of protoplasmic concentration. Therefore, we are unable to say definitely whether the adjusted amoebae were isotonic, slightly hypotonic, or hypertonic to the culture medium. Since the amoeba can live as well in 50 per cent sea water as it can in 5 per cent, and since the protoplasm of amoebae living in 50 per cent assumes a concentration greater than that of 40 per cent sea water, a wide range of protoplasmic concentrations appears to be compatible with the metabolic processes.

Factors affecting the activity of the contractile vacuole system

Contractile vacuoles form and are expelled in all concentrations of sea water in which the amoebae will live and reproduce. The rate of formation and expulsion is, of course, slower in concentrated than in dilute culture media. On encystment, growth and expulsion of contractile vacuoles cease and are resumed when excystment occurs. Kitching (1938) has shown that the oxygen tension of the medium is an important factor in the activities of the vacuoles of some protozoa, including various Peritrichs, *Amoeba proteus*, and *Amoeba mira*. During the present experiments, it has been observed that if the amoebae are sealed under a coverslip and left for some time, the rate of vacuolar expulsion is invariably retarded. On the other hand, if fresh aerated culture fluid is continuously circulated under the coverslip, the vacuoles remain active. This would suggest that oxygen is important in the activity of the contractile vacuoles. More precise experiments on the effect of oxygen are necessary.

A study of the effect of the hydrogen ion concentration of the medium was made. Sudden increases and decreases cause an increase in the rate of vacuolar activity. On the other hand, if amoebae are adjusted to and living at a given pH, there is no consistent relation between the pH of the medium and the rate of fluid elimination.

The effect of the concentration of the culture medium on the activity of the contractile vacuoles

An extensive study was made of the relation between the concentration of the culture medium and the rate of fluid elimination by means of the contractile vacuoles. In view of the fact that uncontrollable factors act to affect the activity of the

vacuoles, the study had to be statistical. For these experiments amoebae were inoculated into cultures made by adding six grains of wheat to various percentages of sea water in Petri dishes. The rate of elimination was measured only after adjustment evidenced by growth and reproduction was complete. Measurements were made of the rate in amoebae living in 5, 10, 15, 20, 25, and 50 per cent sea water. As we have seen, this amoeba flourishes equally well in all of these concentrations.

The rate of fluid elimination was measured as follows: A coverslip was cleansed and dropped onto the bottom of a culture dish; the culture fluid was agitated with a pipette and the amoebae were allowed to settle and attach themselves onto the coverslip. When attachment had occurred, the coverslip was then inverted and placed on a slide between two parallel streaks of vaseline. The streaks of vaseline served to stabilize the coverslip, prevent the amoebae from being crushed between slide and cover, and allow free circulation of water underneath. During an experiment, fresh aerated sea water of the same strength as the culture medium of the particular amoebae being observed was continuously added at one end of the cover and drawn out at the other by means of a strip of filter paper. Observations were made under an oil immersion lens. The diameters of vacuoles were measured with a micrometer eyepiece and the volumes calculated. The vacuole was always measured just before expulsion. The fluid elimination of an amoeba for a ten minute interval was obtained by adding the volumes of the vacuoles formed during that time. The amoeba was then mechanically agitated, causing it to round up, and its volume obtained by measuring its diameter while thus rounded. The rate was calculated in terms of the cubic micra eliminated per minute by 100 cubic micra of protoplasm.

The results of this study of the rate of elimination of amoebae living and reproducing in various concentrations of sea water are presented in Figure 5. The rates of over one hundred amoebae were measured to obtain the curves in this figure. The rate of each amoeba measured is represented by a point. The average rate of all the amoebae measured in each percentage of sea water is represented by a square, while the highest rate obtained for each concentration of medium is represented by a circle. The average rates have been connected by a curve, as have the maximum rates. A third curve has been drawn whose shape is determined by the formula: $RC = K$, where R is the rate of elimination, C is the concentration of the medium to which amoebae are adjusted, and K is a constant.

If we take into consideration cysts whose rates are zero and which are not included in the figure, we find that the rates of amoebae grown in any concentration may vary from zero to a maximum. The maximum rates vary inversely with the concentration. Neglecting those amoebae, or cysts, whose rates are zero, the average rates also vary inversely with the concentration. Neither the curve for the maximum rates, nor that for the average is exactly parallel with the curve expressed by the equation $RC = K$, but the parallelism in both cases is sufficient to justify the conclusion that under ideal conditions in which concentration is the only variable, the equation would express accurately the relationship existing between the concentration of the medium and the rate of elimination.

Kitching (1938) has pointed out that if a high unalterable protoplasmic concentration was maintained by the action of the contractile vacuoles, the rate of

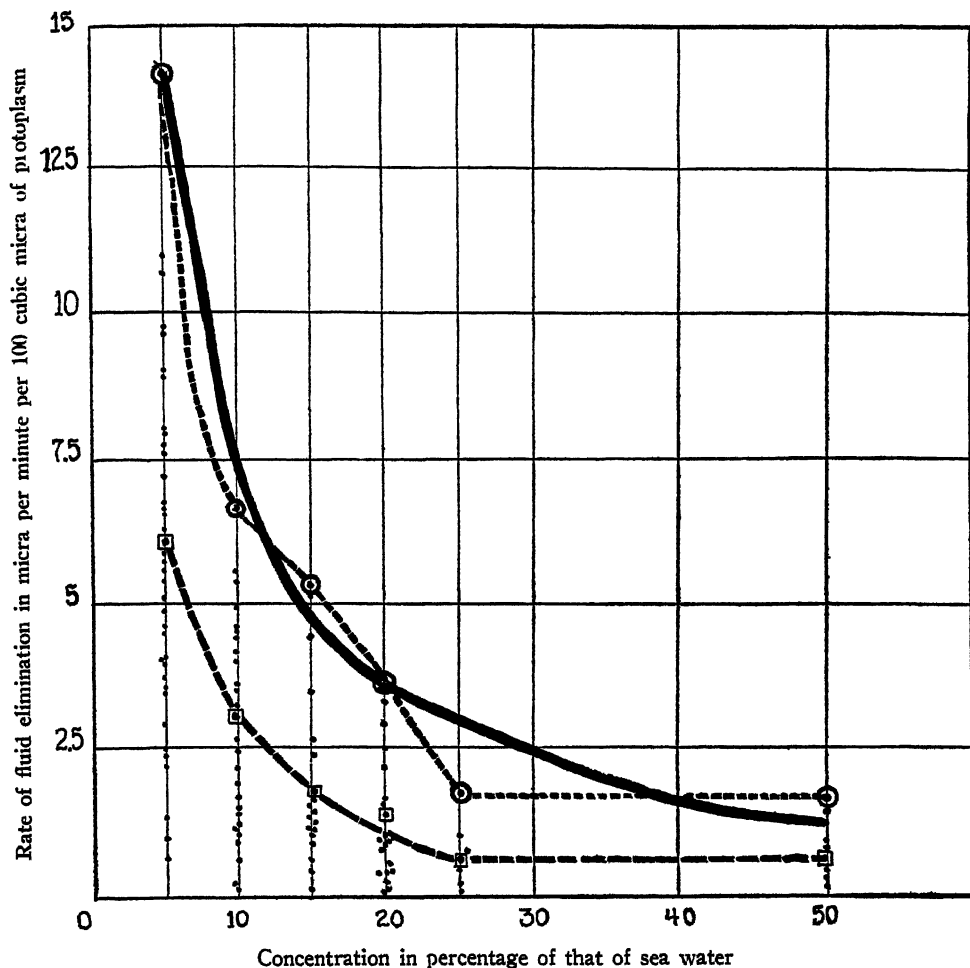


FIGURE 5. Graph showing the relation of the rate of fluid elimination to the concentration of salts in sea water. The amoebae were tested in the concentration in which they were grown. Squares, average rates; circles, maximum rates; dots, individual rates. Solid line is curve for equation; $R \text{ (rate)} \times C \text{ (concentration)} = K \text{ (constant)}$.

elimination should be inversely proportional to the external concentration, and that the curve should be a straight line whose slope is constant instead of a curve whose slope increases as the external concentration decreases. The curves obtained by Kitching (1938) for *Peritrichs*, those obtained by Mast and Hopkins (1941) for *Amoeba mira*, and those obtained here, never approach a straight line. The actual rates are either always too low in the intermediate concentrations or too high in dilute and concentrated solutions, depending of course upon the rate which is assumed to be correct. Therefore, in view of these facts, it is not possible that the correct curve is a straight line.

The effect of changing the external concentration on the volume of individual vacuoles

(A) Vacuoles remaining in the amoeba: When an amoeba with a large contractile vacuole is adjusted to 5 per cent sea water and is placed suddenly in 100 per cent sea water, the amoeba shrinks. By careful observation it can also be observed that the contractile vacuole shrinks. The membrane of a vacuole for a while shows sufficient elasticity to maintain a spherical shape, but the shape is not maintained. As more and more water is drawn out of the vacuole, the membrane collapses (see Fig. 6).

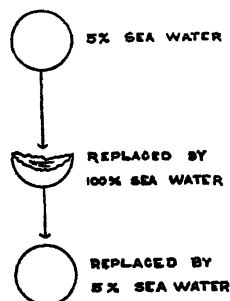


FIGURE 6. Diagram illustrating the collapse of a contractile vacuole when subjected to hypertonic sea water, and its recovery when placed in sea water to which it originally was isotonic.

If the amoeba, now in 100 per cent sea water with the vacuole in the condition shown in Figure 6, is returned to 5 per cent sea water, the amoeba and the collapsed vacuole both absorb water and swell. The vacuole soon regains its original volume and may even become larger. It would appear from this experiment that the vacuole is acting as a simple osmometer. When the hypertonic sea water was added, the water was extracted from the vacuole but not the dissolved substances. Consequently, when dilute sea water was again added this substance acting osmotically drew water back into the vacuole.

(B) Vacuoles removed from the amoeba: In the preceding experiment, it could still be argued that the swelling of the vacuole was due to a secretory process requiring oxygen (Kitching, 1938), since the swelling of the vacuoles took place in the amoeba and in the presence of the cellular activities. If, however, the vacuole was removed from the cell and suspended in the medium free of all contact with protoplasm the influence of other cell parts would be eliminated.

By crushing amoebae under a coverslip while watching the vacuoles closely it was observed that the vacuoles do not always disintegrate; some of them remain intact after they are liberated, free of protoplasm, into the medium. When the vacuoles are thus exposed to the outside medium they swell noticeably, indicating that the elastic forces of the cell were inhibiting swelling to some extent. When such isolated contractile vacuoles from amoebae adjusted to 5 per cent sea water are changed to 100 per cent, they shrink. Then, on being returned to 5 per cent sea water, they swell just as they do while still in the amoebae. *It is obvious then that they act as simple osmometers; that their contents contain osmotically active sub-*

stances in solution, and that the swelling of the vacuoles does not depend upon other protoplasmic processes.

The adjustment of Amoeba lacerata to changing concentration

We have described the immediate effects upon the amoeba and contractile vacuole of changing the concentration of the medium. The story is not completed, however, until we have described the complete series of changes in volume and in the rate of elimination which occur during the adjustment process. Observations have been made upon the volume and vacuolar activities during the adjustment process, both when amoebae cultured in 5 per cent sea water were changed to 50 per cent and when amoebae cultured in 50 per cent sea water were changed to 5 per cent.

(I) Adjustment of amoebae cultured in 5 per cent sea water to 50 per cent sea water: When an actively moving amoeba, raised in 5 per cent sea water, is changed to 50 per cent sea water and allowed to remain, it rounds up and its contractile vacuole shrinks greatly. The amoeba remains for some time in the inactive spherical condition. The contractile vacuole increases in volume very slowly. Repeated measurements of the diameter of the amoeba itself reveals the fact that it increases slowly in volume. This increase continues until it has regained approximately the value it had in 5 per cent sea water. As soon or soon after it has regained this original volume, protoplasmic streaming becomes more definite, attachment to the substratum is made, and normal locomotion begins. The rate of elimination of fluid by the vacuole remains at a low level. In Figure 7*A* and *B* are plotted curves showing the observed changes in protoplasmic volume and the rate of elimination of two amoebae when they were suddenly changed from 5 per cent sea water in which they had been cultured to 50 per cent sea water. In these experiments the rate of fluid elimination by the contractile vacuoles was measured, 50 per cent sea water added, and the volume and rate measured again. Since the rate of elimination was so extremely slow it was possible to make only one measurement before adjustment was completed, but the diameter of the amoeba was measured at frequent intervals until adjustment was complete and locomotion resumed.

(II) Adjustment of amoebae cultured in 50 per cent sea water to 5 per cent sea water: In Figure 7*C* and *D*, we have plotted curves showing observed changes in volume and the rate of elimination when amoebae were suddenly changed from their 50 per cent sea water culture to 5 per cent sea water. In these curves it will be observed that (1) the amoebae swell and then shrink until their original cultural volume is regained, (2) the rate of elimination increases rapidly to a high value which may either remain high, or after an initial increase may fluctuate, and (3) that the time required for the amoebae to regain their original volume may be long or short.

In this experiment it appears quite possible that the activity of the contractile vacuole may have been helpful in bringing the volume of the amoeba back to normal. But we already know from experiments that when adjustment is complete the amoebae will now shrink if placed in 10 per cent sea water. Therefore, while the activity of the contractile vacuole may have helped the amoebae to regain their

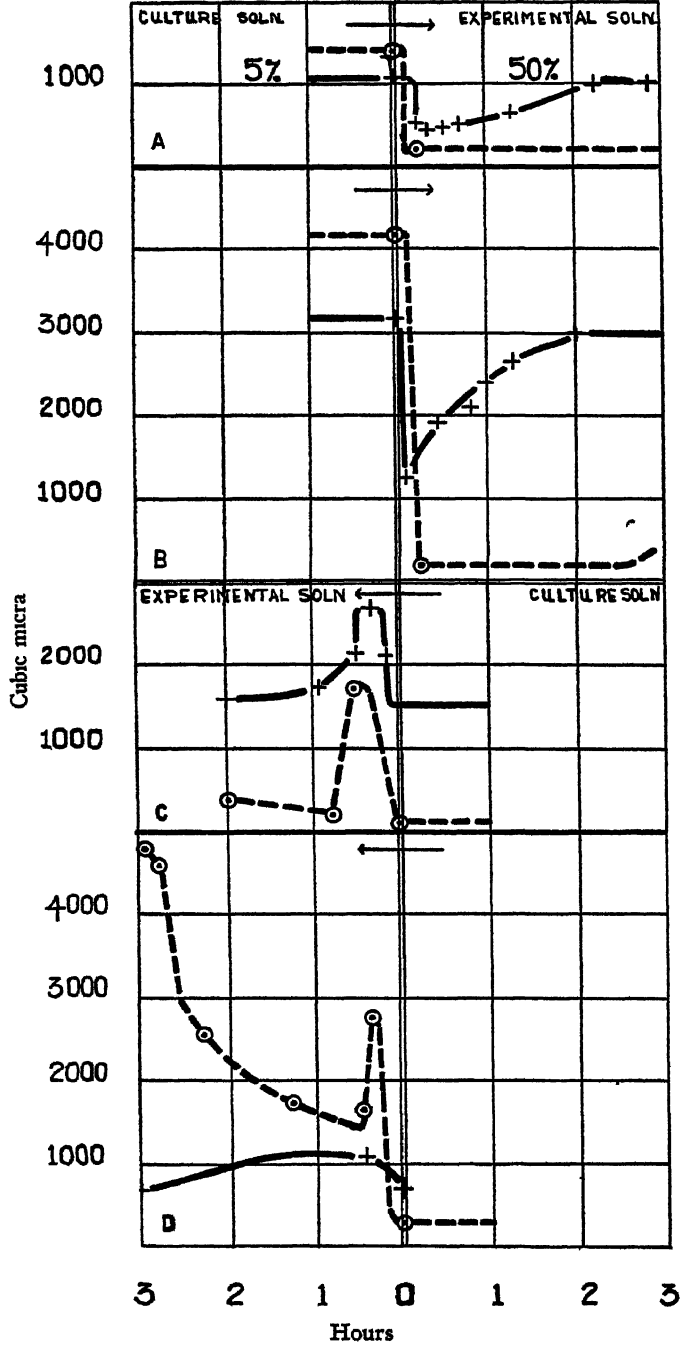


FIGURE 7.

normal volume, the protoplasmic concentration at the same time has dropped from a value approximating that of 50 per cent sea water to a value approximating that of 5 per cent sea water.

DISCUSSION

Krogh (1939) after reviewing the works of various authors on the functions of the contractile vacuoles of the Protozoa, concluded that the evidence was predominantly in favor of the "osmoregulatory theory," which holds that these vacuoles serve to maintain the internal protoplasmic concentration at a constant level which is higher than that of the outside medium. In view, however, of the work of Buchthal and Peterfi (1937) who found only slight potential differences existing between the protoplasm of *Amoeba sphaeronucleus* and the outer medium, Krogh concludes his discussion with a "warning against too schematic conceptions." He says further, "It seems possible that the contractile vacuole may come much nearer in its function to the kidney of higher animals than is indicated by direct studies of osmotic regulation."

The contents of the contractile vacuoles

Weatherby (1927) was unable by means of very delicate tests to show the presence of urea or ammonia in the contractile vacuoles of paramecia. Kitching (1938) points out that the maintenance of a constant concentration higher than the outer medium would require the elimination of fluid more dilute than the protoplasm. He believes from his experience that it is entirely possible that these vacuoles contain only distilled water. Ludwig (1928) argues that carbon dioxide is eliminated by way of the contractile vacuoles, but without evidence. The possibility of the elimination of carbon dioxide, carbonic acid, and carbonates by the contractile vacuoles, however, has not been disproven. Various authors have maintained that granular anlagen give rise to contractile vacuoles (Lloyd and Scarth, 1926; Taylor, 1923; Howland 1924a; MacLennan, 1933). Hopkins (1938) and Mast and Hopkins (1941) maintain that the food vacuoles of the marine amoeba, *Amoeba mira*, in addition to being the seat of digestion, act as do contractile vacuoles and that the anlagen of these vacuoles may contain granules. They also show that the substances that make up these granules may never aggregate in the form of granules but remain in solution in the vacuoles or their anlagen. The present observations on *Amoeba lacerata* do not show definite connections between granules of the cytoplasm and the contractile vacuoles or that their anlagen contain granules, but they do show that they contain osmotically active substances.

The concentration of the protoplasm

The actual concentration of the protoplasm has never been ascertained. When we speak of the osmotic strength or activity of a cell we can of necessity refer only to the attraction of the cell for the solvent and compare the strength of this attrac-

FIGURE 7. Graphs showing changes in volume and rate of fluid elimination of individual amoebae when they were transferred from the percentage of sea water in which they were grown to lower or higher percentages. *Solid curves*, volume changes; *broken curves*, changes in rate of fluid elimination. Arrows show the direction of the concentration changes. *A* and *B* from 5 per cent to 50 per cent sea water; *C* and *D* from 50 per cent to 5 per cent sea water.

tion to the attraction exerted by known solutions. A cell is said to be isotonic to a solution of known strength when the affinity of the cell and the solution for solvent are equal; i. e., a further increase in the strength of the solution would shrink the cell. No deductions can be made as to the total molecular concentration of the cell components. The osmotic concentration, the intermolecular attractions and repulsions of the protoplasmic molecules and tendencies of the protoplasm to become hydrated are all involved. The tensile or elastic strength of the protoplasm, membranes, and cell wall all work in opposition to the osmotic concentration and hydration. The shrinkage or swelling of a cell depends upon a summation of these inside forces. Most cells are in equilibrium with their environment; i. e., the summation of forces inside equals those outside. The cells forming contractile vacuoles are said to be exceptions according to the osmosis-secretion theory of Kitching (1938). Cells forming contractile vacuoles live in media much more dilute than solutions necessary to plasmolyze them. Such cells must be absorbing water continuously from their more dilute outer medium and continuously bailing out fluid, more dilute than the protoplasm, by the action of the contractile vacuole. Even though the concentration of the protoplasm is higher than the outside medium, this does not follow. The elastic or tensile strength of the membrane and ectoplasm or plasmagel, and for that matter intermolecular attractions of the protoplasmic molecules must exert considerable opposition to the entrance of water. The results here presented and those of Mast and Hopkins (1941) show that *the osmotic activity involved in collecting water eliminated by the contractile and food vacuoles is resident in the vacuoles themselves not in the surrounding cytoplasm*. The surrounding cytoplasm serves merely as a membrane through which the water is drawn. It is also clearly seen from these results that we cannot speak accurately of the osmotic activity of the protoplasm in general. Only of specified parts which have no internal differentiation can we hope to accurately be specific as to osmotic activity.

The concentration of the protoplasm of *Amoeba lacerata* is only slightly different from the surrounding medium. The present experiments demonstrate this fact. (see Figures 4 and 7). The results presented in Figure 5 are consistent only if we assume that the concentration of the protoplasm outside of the vacuoles varies in the same direction as the medium. If we also assume that the protoplasm by oxidative or other metabolic processes gives rise to a constant supply of metabolic by-products for isolation into the vacuoles which swell and coalesce in a constant manner (which is true in the average amoeba), then the curve of Figure 5 is explained perfectly. It is unfortunate that the experiments on the rate of elimination could not be continued below 5 per cent sea water. The reason for this inability was the extreme variability of rates obtained. In other words, in extremely dilute media, the rate of elimination showed little or no dependence upon the osmotic pressure of the external medium. This leads to the conclusion that in extremely dilute media, the osmotic pressure of the external medium is of little consequence in determining the rate of fluid elimination.

The function of the contractile vacuoles

If water is absorbed mainly by the cell due to the osmotic activity of the contents of the contractile vacuoles, the primary function of the vacuoles is not the elimination of water. *The water eliminated by the vacuoles is only water absorbed as a*

consequence of their own contents. The osmotically active contents of the vacuoles are discharged to the outside and are wasted. These wastes are in all probability metabolic by-products. They could be incompletely oxidized food, carbonic acid, carbonates, ammonia, urea, uric acid, or other waste products.

Kitching (1938) has shown that activities of contractile vacuoles and volumes of various protozoa are controlled by the oxygen tension. He takes this fact to mean that oxidative energy is utilized in extracting water from the protoplasm and the secretion of this water into the vacuoles. He argues that energy is needed since this is accomplished against an osmotic gradient. He also finds that cyanide inhibits the action of the contractile vacuoles in these forms and that coincident with cyanide inhibition, the body volume increases. When cyanide is removed, the contractile vacuoles resume their activity and the cell volume returns to normal. His interpretation of these facts is that the removal of oxygen or the addition of cyanide inhibits oxidation which in turn deprives the vacuoles of energy and they cease extracting water from the protoplasm; but meanwhile the cytoplasm continues to absorb water from the medium thus swelling the cell. He omits any consideration of the effect of cyanide on protoplasm except the inhibition of oxidation. His interpretation does not take into consideration the following possibilities: (1) While the relation of oxygen tension to vacuolar activity is demonstrated, this relation could be as well explained by the formation of osmotically active substances in vacuoles as a result of oxidation. (2) Cessation of the oxidative process may initiate many changes in the protoplasm, for example, its degenerative breakdown which would cause swelling. Kitching's own results, the results of others, and the present results are more consistent with the conclusion that the contractile vacuoles function to eliminate metabolic wastes, and that the elimination of water is merely a consequence of the osmotic activity of these wastes.

When one stops to consider the possibility of the formation of vacuoles more dilute than the protoplasm, it is seen to be an impossibility and contrary to known physico-chemical laws for the following reasons. For it to be possible, water would have to be isolated and separated against solution and chemical forces. This water would have to be isolated into regions of equal hydrostatic pressure by some force which cannot exist since water has been attracted into the protoplasm by exactly opposite forces. There are two ways by which water can be separated from protoplasm, i.e., setting up forces which attract or repulse water more strongly than protoplasm. (1) By chemical changes occurring in regions of the protoplasm, for instance, oxidation which results in localized increases in chemical and osmotic forces (vacuoles). (2) By changes in protoplasm in general. Energy resulting from the oxidation of food can be used in polymerization whereby the forces leading to hydration are lost. This releases water from combination with the protoplasm. The water would tend to collect in localized regions, but during this collection, waste products become dissolved and consequently, the vacuoles so formed would contain waste and salts, not distilled water. Oxidation in the protoplasm enhances the water-combining powers of some substances, but not all.

If the vacuoles contain a solution whose osmotic pressure is less than that of the surrounding cytoplasm, then water will be drawn back into the cytoplasm and not expelled to the outside. This statement, however, would not be true if the vacuole membrane were impermeable to water.

In the experiments concerned with isolated vacuoles, it has been clearly shown that the internal contents of the vacuoles do have an osmotic pressure and that the vacuole membrane is permeable to water. *The osmotic pressure of the vacuoles is either equal to or greater than that of the cytoplasm.*

The relation between the osmotic activity of the protoplasm as a whole and that of the external medium in which the amoebae live

We have demonstrated that the osmotic activity of *Amoeba lacerata* varies directly with that of the environment. This also is true of *Amoeba mira* (Mast and Hopkins, 1941) and of the peritrichs investigated by Mast and Bowen (1945). In addition, it appears that *Amoeba proteus* (Mast and Fowler, 1935), peritrichs (Mast and Bowen, 1945), plant cells (many authors) and many other cells maintain a higher osmotic pressure than their natural medium. This is the cause of turgidity. In the protozoa with contractile vacuoles as well as other animal and plant cells, the extent of this hypertonicity of a cell over its environment must depend upon the strength of the membranes, and other ectoplasmic structures. The external medium remaining constant, some of the factors that would cause swelling of cells are: (1) weakening of cell boundaries, (2) peptization of protoplasmic proteins, (3) increase in cellular anabolism without increase in cellular excretion, or (4) merely failure of the excretory processes.

The extent of the difference in osmotic pressure existing between the cell contents and the outside medium has not been demonstrated either for *Amoeba mira* or *Amoeba lacerata*, but if we include consideration of the osmotic pressure of the vacuoles, then the difference is appreciable but variable, being higher inside than outside.

Factors determining the rate of vacuolar output

In a given protozoan, the rate of fluid output depends on a number of factors. These factors and how they operate are not clearly defined but the following undoubtedly are involved:

(1) Respiration. The work of Kitching has demonstrated this. If the basic osmotic pressure of a cell is in equilibrium with that of the environment, the oxidative (aerobic or anaerobic) breakdown of foodstuffs will result in an increase in the osmotic activity of the cell as a whole even though these by-products are probably isolated into the vacuoles immediately by the formation of a precipitation membrane. It is readily seen from the facts presented here and by Mast and Hopkins (1941) that the rate of fluid elimination would depend upon the respiratory rate. Condensation of the by-products in the form of condensation granules would decrease their osmotic activity. While the rate of oxidation does have this controlling influence upon the rate of fluid elimination, the shape of the curve of Figure 5 is not determined by variations in oxidative rate. The oxygen supply, of course, was not absolutely constant but reasonably so. Since the curve represents an average, the variations are accounted for statistically.

(2) Food. In protozoa, food taken into food vacuoles and digested there adds to the over all osmotic activity of the cell, but if digested food is rendered insoluble by condensation into crystals or other food reserve bodies, this osmotic increase is nullified. This storage may not take place before appreciable increase in intake of

water has been affected. Also, the water engulfed with food would naturally have an influence on the rate of fluid uptake, but not upon the output as suggested by Kitching (1939).

(3) Agents causing polymerization or breakdown of substances in the protoplasm.

(4) Changes in osmotic activity of outer medium will cause increases or decreases in water intake and consequently the output. The permanence of these effects will depend upon the permeability of the membrane to the substances in the external medium.

Two vacuole systems as opposed to one

Amoeba mira (Hopkins, 1938) forms only one system of vacuoles. By this statement it is meant that all vacuoles which arise in this amoeba coalesce to form large cloacal vacuoles, which are eliminated in order of formation. The functions of excretion and digestion are accomplished by this system. On the other hand, *Amoeba lacerata* forms two entirely different sets of vacuoles: one which goes to make up the contractile vacuoles, and is excretory in function while the other system goes to combine with the engulfed food and is digestive in function. All protozoa probably can be separated into two groups: one primitive group, having only one vacuolar system; and a specialized group, having two systems.

SUMMARY

1. *Amoeba lacerata*, a fresh water amoeba, is able to adjust and live in any concentration of the salts of sea water up to 125 per cent.

2. Contractile vacuoles are formed in all concentrations and are separate from food vacuoles.

3. Food is engulfed with little or no water and small protoplasmic vacuoles coalesce with it. When digestion is completed, the food residues are eliminated with little or no fluid.

4. The major part of fluid elimination occurs by way of the contractile vacuoles.

5. Contractile vacuoles grow in size by coalescence and osmotic swelling.

6. The rate of fluid elimination is affected by a number of factors, but under constant optimal conditions it varies inversely with the concentration of the medium. It is suggested that the rate is proportional to the rate of catabolism.

7. The osmotic pressure of the protoplasm varies in the same direction as that of the outside medium. The protoplasm of completely adjusted amoebae is very nearly equal to that of the medium, the osmotic difference being less than that of 5 per cent sea water (0.13 atmosphere).

8. Contractile vacuoles have been shown to contain osmotically active substances; they shrink in hypertonic and swell in hypotonic solutions regardless of whether they remain in the cell or have been removed from the cell.

9. The volume of the amoeba is only temporarily dependent on the concentration of the medium. After adjustment, the volume shows no dependence upon concentration of the medium. If the amoeba is placed in a hypertonic solution the cell shrinks and then swells to its original volume within three hours time. Conversely, if placed in a hypotonic solution, it swells and then shrinks to its original volume.

10. The cell membrane is either permeable to substances in the medium especially when adjusting to a change in the concentration of the medium, or is capable of modifying its internal osmotic activity in some other way.

11. The contractile vacuoles are obviously excretory and also osmoregulatory organelles since they remove waste substances which would otherwise cause increases in the osmotic pressure in the cell

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THE BIOLOGICAL BULLETIN

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DILUTION MEDIUM AND SURVIVAL OF THE SPERMATOZOA OF *ARBACIA PUNCTULATA*. II. EFFECT OF THE MEDIUM ON RESPIRATION

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INTRODUCTION

The first section (Hayashi, 1945) of this investigation presented evidence for the existence of a factor in the seminal fluid of *Arbacia* which influences the duration of fertilizing capacity of the sperm of the same species. The experimental results indicated that the factor was adsorbed on the sperm surface, and subsequently was lost into the surrounding medium. Since Gray (1928a) has shown a relation between "mechanical crowding" of sperm and the length of metabolic life of sperm, the question at once arose as to whether the seminal fluid factor influenced the fertilizing power of sperm through an effect on the metabolism of sperm. Consequently, a series of experiments was done investigating the effects of seminal fluid on the respiratory activity of sperm.

MATERIALS AND METHODS

The microrespirometer used for these experiments was a compensating type, a modification of Krogh's respirometer.¹ It consisted of two similar vessels of conventional design connected through a common U-shaped manometer. To remove the carbon dioxide, filter paper wet with 20 per cent KOH was placed in the manometer vessels out of contact with the respiring cells. The manometer vessels were immersed in a constant-temperature bath maintained steadily at 25° C. The shaker, upon which the microrespirometers were mounted, moved to and fro in a straight line a distance of 15 centimeters. A steady rate of 40 cycles per minute thoroughly agitated the respiring sperm suspensions. The sensitivity of the respirometer was such that a respiratory rate of one mm.³ of oxygen consumed per hour could be detected in ten-minute readings.

Two points of difficulty were encountered in the course of these experiments. First, capillary action prevented the easy transfer of any liquids from the side-arm into the respirometer vessel. Second, the effect of dilution on the respiration of sperm was found to be very rapid, and therefore difficult to measure. To overcome these difficulties, a measured amount of packed sperm was placed on the glass wall

¹ Kindly lent by Dr. Titus C. Evans, formerly of Iowa State University.

of the manometer vessel above the point reached by a given amount of liquid in the vessel, when this liquid was agitated by the movements of the shaker. The packed sperm remained in place by their own adhesive action. When the time came to dilute the sperm in the medium to be studied, the adhered sperm on the side of the vessel were washed down into the liquid by a slightly greater agitation of the manometer vessel.

This technique overcame the first of the above-mentioned difficulties satisfactorily, but only partly the second. To wash down and to disperse completely the sperm cells in the medium required time in the order of minutes. Since, during this period, changing numbers of cells were being affected by the medium, neither the total respiration nor the total respiratory rate could be accurately measured. This effect was minimized by manipulation of the vessels and by extrapolation of the respiration curve.

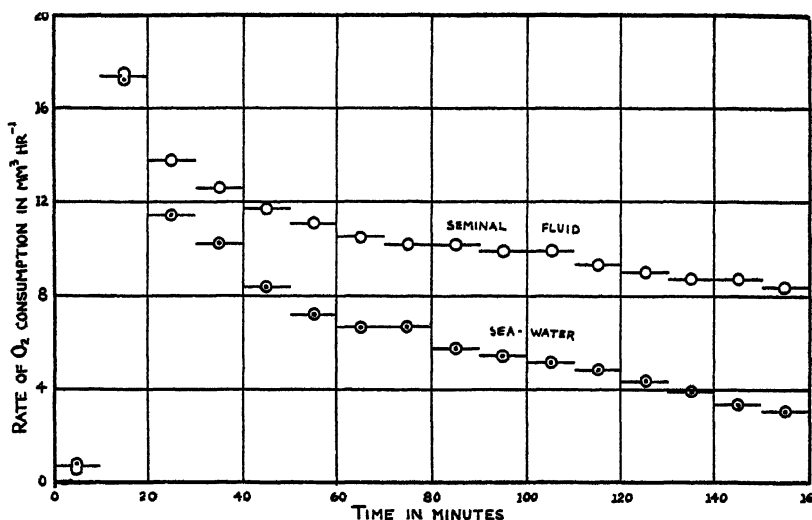


FIGURE 1. Effect of seminal fluid, sea water on respiratory rate of sperm.

The pH of the medium in the experiments here presented was not checked after each run, although it was known before the run, and has been published elsewhere (Hayashi, 1945). In later, similar experiments (to be published) the pH was adjusted to equality and checked before and after each run. In the time of the run, no change in pH occurred, and the results did not differ from those presented.

It was noted that the sperm in all the respiration experiments, irrespective of the medium used, and in the concentrations of these experiments, were dead at the end of five hours. A sharp rise in the rate of oxygen consumption occurs at about this time, due, probably, to disintegration of the sperm cells caused by the constant shaking of the manometers. This same effect was noted by Gray (1928b, p. 350). This effect afforded no trouble in the interpretation of the results, however, for it occurred uniformly in all sperm suspensions, and the pertinent data were obtained before the end of the five-hour period.

EXPERIMENTS AND RESULTS

For the first experiments, the respiration of sperm in seminal fluid was compared with the respiration of an equal amount of sperm in sea water. Each suspension contained 0.00155 cc. of packed sperm per cc. of medium. The contrasting changes in the respiratory rates of the two suspensions are shown in Figure 1. Using the same data, the total oxygen consumed was plotted as a function of time, to produce the curves of Figure 2.

The experiment showed that the seminal fluid did not affect the sharp increase in respiratory activity immediately following the dilution of the sperm. The main effect of the seminal fluid was to regulate, or delay, the sharp drop in respiratory rate shown by the sperm in sea water. Figure 1 also showed that the total amount of energy expended by equal amounts of sperm in the same length of time was greater when the sperm were suspended in seminal fluid.

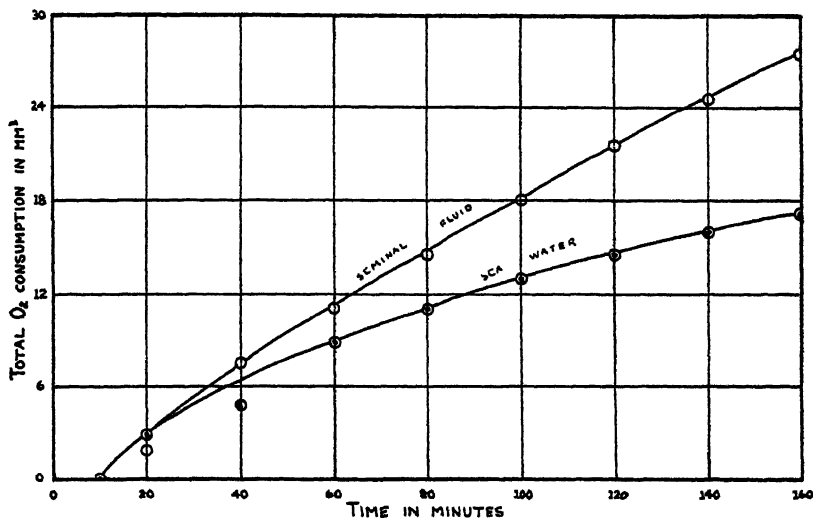


FIGURE 2. Total oxygen consumption as a function of time for sperm in seminal fluid and sea water.

As a further check on the effect of dilution on the respiration of sperm, one of Gray's (1928a) experiments was repeated, with both seminal fluid and sea water. A measured quantity of packed sperm (0.00155 cc.) was placed on the wall of the respiration chamber, and 1.0 cc. of medium was placed on the bottom of the vessel. In addition 0.5 cc. of medium was placed in the side arm, and this amount of medium was "dumped" over into the respiration chamber at an appropriate time. The results of the experiment are shown in Figure 3.

The results confirmed Gray, but compared with his results, the degree of rise in the respiratory rate at the second dilution was less. When the sperm concentrations used by Gray were compared with those used here, it was seen that the present sperm suspensions were far more dilute. Therefore, the results were taken to mean that the concentration of sperm in this experiment was near the

upper limit to obtain the maximum burst of energy at first dilution from the amount of sperm used. In other words, greater original dilution of the packed sperm would not result in very much more initial activity.

This interpretation was partly confirmed by the following experiment. Lillie (1913) had found that egg water stimulated sperm to greater activity, and Gray (1928c) concluded from his experiments that egg water stimulated sperm to greater respiratory activity. However, Gray had used egg water as the first and only diluent for the sperm. In the following experiment, by contrast, the egg water was used as a diluent after the initial burst of activity induced by the original dilution of the medium.

The same procedure as the preceding experiment was employed. Instead of the respective media in the side arms, however, egg water was used, and this was

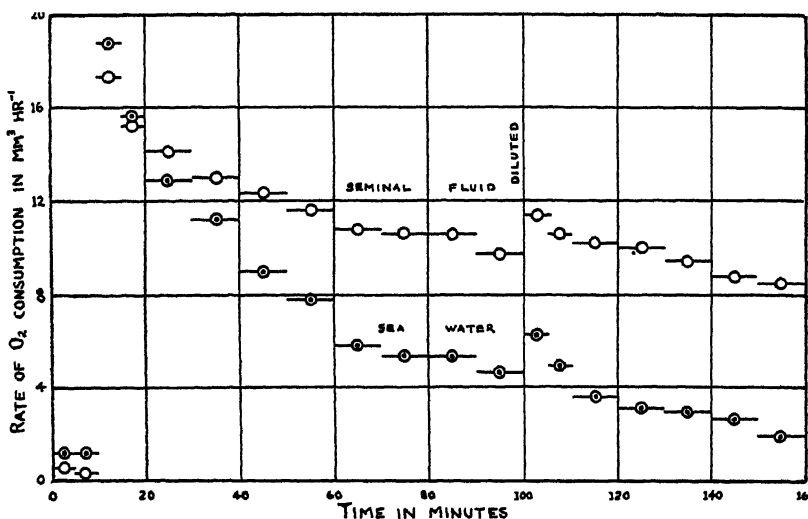


FIGURE 3. Effect of dilution by respective media on sperm in sea water and seminal fluid.

"dumped" in at the appropriate time. The results are given in Figure 4. Study of Figure 4 showed that, after the original burst of activity, egg water, far from stimulating the sperm, seemed to inhibit them. The effect was probably due to the agglutination of the sperm, and seemed to overcome the slight stimulation due to dilution shown in Figure 3.

DISCUSSION

Analysis of the effect of the seminal fluid on sperm respiration

The respiratory rate of sperm in seminal fluid is compared with that of sperm in sea water in Figure 1. The seminal fluid does not seem to influence the initial steep rise in activity due to dilution. There was some variation shown in this first burst of respiration, but a check of all the runs made showed that the variations were not significant. In some cases the sperm in seminal fluid showed more intense

activity at dilution; in others, the sperm in sea water were more active at dilution. The variations were caused probably by differences in the rate at which the sperm were washed from the sides of the respiratory vessels.

The effect of the seminal fluid is on the "senescent period" following the initial respiratory rise. The seminal fluid seems to prevent the rapid decline of the metabolic rate evident in the sea-water control. The effect of the seminal fluid on the respiration of sperm thus parallels the effect of seminal fluid on the fertilizing power of sperm. In both cases, the seminal fluid maintains the sperm at a high functional level for a longer time than does sea water. A comparison of the areas under the curves shows that sperm in seminal fluid expend more energy than an equal amount of sperm in sea water during the course of their active lives. There are two possible explanations for these results. It is possible that the sperm in seminal fluid utilized

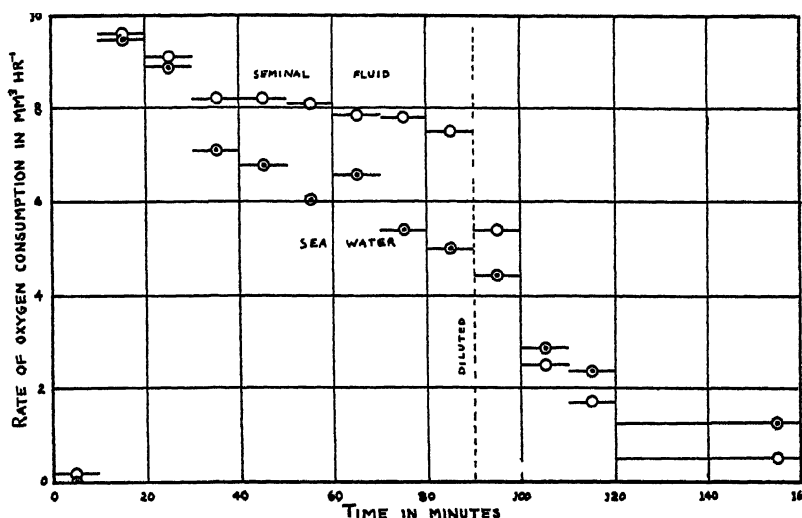


FIGURE 4. Effect of dilution by egg water on sperm in sea water and seminal fluid.

their internal store of fuel more completely than the sperm in sea water. The second possibility is that the seminal fluid is serving as a source of fuel for the sperm. A clue to these possibilities was sought by utilization of some of Gray's analytical methods.

Gray (1928b) originally advanced the view that the sperm cell was a tiny combustion engine with a limited supply of fuel. From his original assumption, Gray derived the equation

$$\text{Activity} = \frac{dx}{dt} = k(a - x) \quad (\text{Equation 1})$$

which, integrated, gave

$$= \frac{1}{t} \ln_e \frac{a}{a - x}. \quad (\text{Equation 1a})$$

In this equation, x represented the amount of fuel used up in time, t ; a , the initial, total amount of fuel in the sperm cell; and k , the activity produced by each unit of fuel consumed. Gray found that this "law of exponential decay of a homogeneous population" did not fit the experimental results. He, therefore, expanded the equation with a consideration of a heterogeneous population, and derived, as one form of the equation,

$$x = O_2 \left(\frac{t}{\alpha + t} \right), \quad (\text{Equation 2})$$

in which O_2 represented the total oxygen consumed during active life; α , another constant; and x and t , possessing the same meanings as before. Gray found that this equation could be made to fit the experimental results, and concluded that the sperm population was heterogeneous in a special way, and that its activity decreased because of the depletion of an internal supply of energy. The notion of heterogeneity was used as a basis for a later, more detailed mathematical analysis (Gray, 1930).

The above methods were applied to the results of a number of experiments of this investigation. At two different values of time t in a single run, experimental values of the total oxygen consumption were arbitrarily selected, and the values of the constants k , a , O_2 , and α in equations 1a and 2 were calculated. The values of the constants thus derived were then substituted back in the equations and the theoretical values of x for the entire run calculated and compared to the experimental values. The results showed that, with the proper selection of points, both equations 1a and 2 could be made to fit the experimental data rather closely, but other points could be selected to demonstrate that neither of the equations fit the experimental data. The latter case, in a typical experiment, is shown in Table I.

TABLE I

Comparison of the experimental and theoretical values of the total oxygen consumption of sperm in sea water and seminal fluid. Theoretical values calculated from equation 1a and equation 2

Total oxygen consumed in mm.³

Time (minutes)	Sea water			Seminal fluid		
	Exp.	$k = .01275$ $a = 12.24$	$O_2 = 15.25$ $\alpha = 71.15$	Exp.	$k = .00876$ $a = 16.31$	$O_2 = 22.54$ $\alpha = 135.7$
0	0.00	0.00	0.00	0.00	0.00	0.00
10	1.05	1.45	1.87	0.95	1.36	1.55
20	2.07	2.75	3.32	1.99	2.62	2.90
30	3.09	3.89	4.50	3.08	3.77	4.08
40	4.06	4.89	5.46	4.06	4.83	5.13
50	4.96	5.77	6.26	5.06	5.78	6.07
60	5.84	6.55	6.94	6.04	6.66	6.91
70	6.68	7.23	7.53	6.97	7.47	7.67
90	8.08	8.36	8.49	8.60	8.90	8.99
100	8.70	8.86	8.88	9.37	9.52	9.56
110	9.23	(9.23)	(9.23)	10.09	(10.09)	(10.09)
210	11.41	11.40	11.37	13.80	13.72	13.69
220	11.50	(11.50)	(11.50)	13.94	(13.94)	(13.94)
255	11.76	11.76	11.90	14.31	14.56	14.71

It may be noted that the points selected are those covering the end of the run, and the data of this portion fit the experimental values rather closely, whereas wide variation is evident in the early portions of the run. Points selected near the beginning of the run, in contrast, would show a fit in that portion of the run, and variation at the end. Intermediate points, of course, would show an intermediate fit.

From such analyses, these conclusions may be drawn. (1) There is no need to assume heterogeneity of the sperm population whether in sea water or seminal fluid. (2) The simple "law of exponential decay" is not adequate to explain sperm activity; the decrease in activity of the sperm cells with time is not due to the exhaustion of an internal supply of fuel alone, whether the sperm cells are in sea water or seminal fluid.

Since sperm cells in sea water show a decline in activity whose controlling factor is not the exhaustion of their internal source of fuel, it is possible that even at the end of active life in this medium, the sperm cells contain unused, potential energy. The utilization of this unused energy by the sperm in seminal fluid would explain the increased oxygen consumption of the sperm cells in seminal fluid. There is, however, the possibility that the sperm in sea water reach the end of active life because of starving *plus* other factors. The question of nutrition by seminal fluid, therefore, is unsettled by the above analyses, although strong possibilities are afforded.

Gray (1928b) analyzed the senescence of spermatozoa from still other considerations. He assumed that the cause of the death of the spermatozoon was the accumulation of products of metabolic activity inside the sperm cell. This would render inactive part of the active system originally present in the cell.

Active system + product of activity \rightleftharpoons inactive system.

Based on this concept Gray derived an equation which he called the "theory of autointoxication."

$$x = \sqrt{\frac{2Ka}{b}} \sqrt{t}. \quad (\text{Equation 3})$$

Here x , a , and t designated the same quantities as before, and K and b were constants. Equation 3, based primarily on the assumption of a homogeneous population, showed that the amount of fuel, x , consumed in time t was directly proportional to the square root of t . Therefore, the total oxygen consumed should show a straight-line relationship to the square root of the age of the active suspension. Gray found that this relationship did not hold for sperm in sea water, for toward the end of active life the points fell below the expected values. He concluded that part of the active system was not only being inactivated, but also being irreversibly destroyed.

Equation 3 was applied to the experimental figures of the present investigation. When the total oxygen consumption of the sperm in sea water is plotted against the square root of the time, the results confirm Gray's findings, for, toward the end of the run, the points fall below the expected values. (The variation from the straight-line relationship at the beginning of the run is due to the time required for the cells to be completely dispersed in the medium.) For the sperm in sea water, therefore, an irreversible destruction of part of the active metabolic system is taking place to-

ward the end of the run (Fig. 5). In contrast, the straight-line relationship is followed for a much longer time in seminal fluid in an equivalent run (Fig. 6). In some of the experiments, a departure from the straight-line relationship occurred in the seminal fluid, but in all cases, this departure occurred very much later from the beginning of active life. In the seminal fluid, therefore, the irreversible destruction of the active system is much delayed, as compared to the case of the sperm in sea water. The decay of activity of the sperm in seminal fluid is apparently due, at least for a great part, to a process of autointoxication.

The difference between the metabolism of sperm in sea water and in seminal fluid apparently lies in the fact that some sort of metabolic system is kept intact for a longer time when the sperm cells are suspended in seminal fluid. The sperm and a factor in the seminal fluid, therefore, constitute a closed system. In sea water,

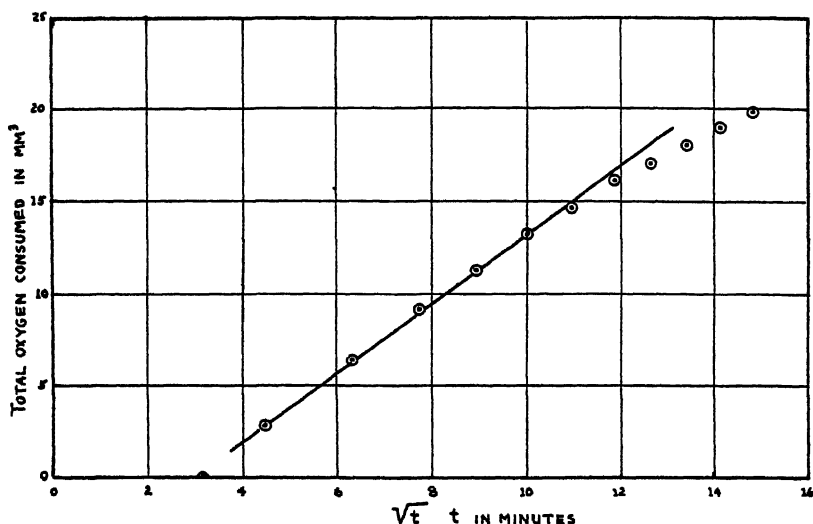


FIGURE 5. Total oxygen consumption as a function of the square root of the time in minutes Sperm in sea water.

part of this closed system is destroyed quite early during the active life of the sperm.

The above findings, supported by the knowledge that seminal fluid contains no reducing sugars (Hayashi, 1945), lends further support to the idea that the effect of seminal fluid on sperm respiration is not due to the replenishment of a store of energy, but rather to the maintenance of a metabolic system. Other experiments (unpublished data) measuring the R.Q. of sperm cells both in sea water and seminal fluid show a characteristic carbohydrate metabolism for these cells, further supporting the contention that the seminal fluid does not provide added nutrients for the sperm cells. It may be concluded that, although there is the possibility that sperm can be nourished experimentally, it is most important for future experimentation that the system, of which the sperm cells are only a part, be kept intact. Workers who have diluted sperm cells in sea water have diluted not only cells, but, also, system.

Both the fertilizing power (Hayashi, 1945) and the respiratory activity of sperm are maintained by a common factor, the seminal fluid. As a first possibility, the role of the seminal fluid factor may be considered fundamental to the respiratory activity only, the fall in fertilizing power being a manifestation of the loss of activity of the sperm cells. In other words, the fall in fertilizing power is only a secondary effect in relation to the seminal fluid factor. The second possibility is that the seminal fluid plays a part directly in both fertilization and respiration. Lack of data does not permit the choice of these possibilities. However, the fact that a seminal fluid factor influences sperm respiration is implicit in both possibilities.

A mechanism, tentatively proposed earlier as the wearing off of a protein substance from the surface of the sperm cell, was adequate to explain the loss of fertilizing power of sperm in sea water. The same mechanism is also applicable to the

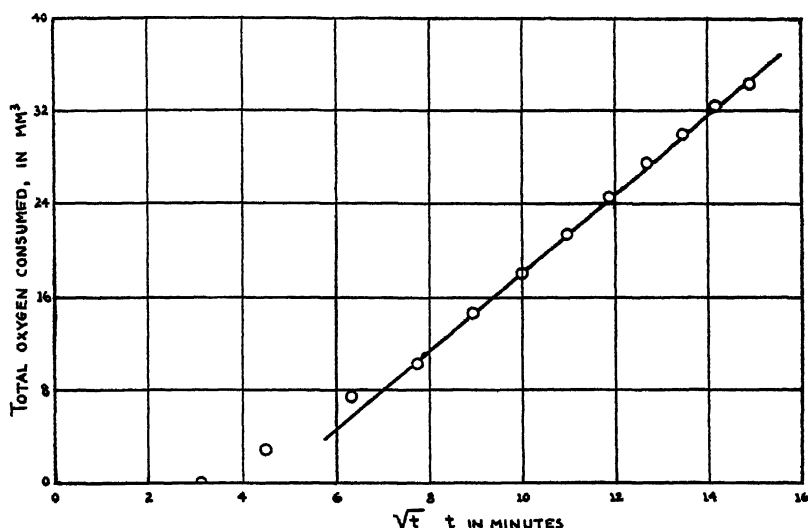


FIGURE 6. Total oxygen consumption as a function of the square root of the time in minutes. Sperm in seminal fluid.

results of respiration studies, if the assumption be made, that the removal of each molecule of protein substance from the surface of the sperm cell releases to the sperm cell a finite amount of the total internal energy. The rate of sperm respiration thus would be controlled by the rate of removal of substance from the sperm surface, which is in turn controlled by other factors. The principal factor affecting the rate of removal of a sperm-surface-substance is the "mechanical crowding" factor studied by Gray (1928a), who found that sperm cells exhibited a burst of activity when diluted. This was confirmed in the present study (Figs. 1, 3, 4). According to the mechanism proposed, the rate of loss of sperm-surface-substance is inhibited when the cells are in a crowded condition, but when relatively far apart, the removal of surface-substance is enhanced.

The removal of sperm substance from the cell surface presumably constitutes the irreversible destruction of part of the active system noted in Fig. 5. In seminal fluid,

however, the replacement of the surface-substance would delay the onset of this irreversible destruction (Fig. 6), the senescence of the sperm cells being conditioned by internal autointoxication, and perhaps also by depletion of the internal energy. It would seem that for the sperm cells in sea water, the destruction of part of the system, in addition to autointoxication and probable depletion of fuel, controls the fall in activity.

It is understood that the mechanism as outlined is tentative at best, but it serves as an explanation of the facts so far known. The facts fit well with the earlier results of the effect of seminal fluid on the fertilizing capacity of sperm. It may be concluded that possibly a common mechanism underlies the effect of seminal fluid on the fertilizing function and the respiration of sperm. The relationship between the surface-substance and the respiratory mechanism of the sperm cell was not investigated. The action of the surface-substance while attached to the sperm cell is possibly enzymatic.

Effect of egg water on sperm respiration

Egg water does not stimulate the respiration of sperm cells but seems to condition a sharp drop in the respiratory rate (Fig. 4). When egg water was added to sperm suspended in sea water and, also, to sperm suspended in seminal fluid, the effect was a sharp decrease in the respiratory rate. This decrease of metabolic activity upon dilution with egg water is in contrast to the effect of dilution with sea water and seminal fluid shown in Figure 3. The increase in respiratory rate brought about by dilution with sea water and seminal fluid shows that even at the dilution used, "mechanical crowding" was still apparently a factor inhibiting the respiration of spermatozoa. Dilution with egg water also relieved the "mechanical crowding," but the agglutinating effect of egg water apparently overcame the effect of dilution so that the respiratory activity decreased.

The stimulation of metabolism by egg water noted by Gray (1928c) and Carter (1930) is not confirmed in these experiments. However, it may be recalled that Carter (1931) had found no stimulation of ripe sperm by egg water. In addition, the egg water of the present investigation was added, not to undiluted sperm, but to sperm that had already been activated by an initial dilution. Therefore, it is possible either that egg water did not affect the respiration of sperm at all, or that the difference in the time of addition of egg water was the cause of the disparity of the results of Gray's experiments and the results of the present investigation.

SUMMARY

1. Seminal fluid has the property of delaying the fall of respiratory activity of the sperm after the original burst of activity upon dilution.
2. Gray's "theory of exponential decay" is not adequate to explain sperm metabolic activity, whereas the "theory of autointoxication" fits the activity of sperm cells suspended in seminal fluid.
3. The seminal fluid delays appreciably the onset of an irreversible destruction of part of the metabolic system.
4. Fertilization studies have led to the formulation of a tentative mechanism based on the adsorption of a protein substance and its removal from the surface of the sperm cell.

5. The proposed mechanism also explains adequately the results of the respiration experiments. Therefore, it is concluded that a seminal fluid factor, by its action while on the surface of the sperm, influences both the fertilizing capacity and the respiratory rate of spermatozoa.

6. Egg water added to a sperm suspension after the original dilution, causes a sharp decrease in the respiratory rate.

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THE EFFECTS OF LITHIUM CHLORIDE ON THE FERTILIZED EGGS OF NEREIS LIMBATA

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INTRODUCTION

The action of the lithium ion on the eggs of certain animals has long been known. Using a number of lithium salts, Herbst (1892) obtained abnormal embryos of two types. In the first group, the endoderm was found not inside the body, but outside; these forms are designated "exogastrulae." Larvae of the second group showed an apparent conversion of ectoderm into endoderm so that in extreme cases, the whole blastula wall was endodermized (Herbst, 1892, 1893, 1943). Herbst concluded that this was a specific and typical effect of lithium, a view shared by Gurwitsch (1895), Morgan (1902), and other early investigators.

Studying the effects of 0.2 per cent to 1.0 per cent solutions of lithium chloride on frog and toad eggs, Gurwitsch found that in the resulting embryos, gastrulation was abnormal, but no exogastrulae were produced. Morgan (1902), using similar concentrations of lithium, obtained embryos in which the black hemisphere had sunk into the interior of the egg, but here again there was no definite indication of exogastrulation. Experiments by Holtfreter (1931) showed that amphibian exogastrulae could be obtained if the blastulae were treated with a modified Ringer solution. Further experiments to ascertain the effects of lithium on the amphibian egg were carried out by Bellamy (1919) who observed a number of cases of fusion of the lateral sense organs of the larvae, as well as abnormalities in gastrulation. Töndury (1938) treated urodele embryos in early stages of gastrulation with LiCl and described a high percentage of head and foregut abnormalities in the larvae. Still another effect of lithium on urodele eggs was observed by Cohen (1938) who treated early gastrulae or late blastulae and observed cases in which the myotomes formed a continuous sheet across the midline, separating the nerve cord and notochord. He also obtained embryos in which exogastrulation had occurred, seemingly as a result of the treatment with lithium.

The effects of LiCl on the eggs of the pond snail, *Limnaea stagnalis*, were studied by Raven (1942) who was able to produce forms which he designates as exogastrulae. He also describes a number of larvae in which varying abnormalities of the eyes were apparent. These experiments are of interest in connection with the present series because the egg of *Limnaea*, like that of *Nereis*, is an example of the so-called "mosaic" type of development.

A wide variety of chemical and physical agents has since been found to produce echinoderm exogastrulae, thus invalidating the theory of an ion specificity. These agents include hypotonic sea water, isotonic solutions of magnesium chloride and lithium chloride, and combinations of isotonic solutions of magnesium chloride, so-

dium chloride, potassium chloride, and calcium chloride as studied by Waterman (1932); solutions of nickel chloride and copper chloride (Waterman, 1937); lithium in low concentrations augmented by carbon monoxide (Runnström, 1935); and 97 per cent carbon monoxide with 3 per cent oxygen in the presence of light (Runnström, 1928a). MacArthur (1924) treated sea urchin eggs with calcium chloride, stale or diluted sea water, copper sulfate, mercuric chloride and potassium cyanide, and obtained exogastrulae. Using "auxin," glycogen and potassium chlorate, Motomura (1934) likewise obtained echinoderm exogastrulae.

Most of the eggs previously tested with lithium have been those of echinoderms and amphibians, both of which are characterized by an invaginative type of gastrulation. It seemed, therefore, that an egg showing a strictly epibolic form of gastrulation should be tested, in an effort to ascertain if the same effect could be produced. The following experiments were performed to study the effects of lithium on the fertilized eggs of the annelid, *Nereis limbata*.¹

METHODS

Gametes of the heteronereis form of *Nereis limbata* were obtained at Woods Hole from June to September, during appropriate phases of the moon. Usually the eggs from one female were sufficient for an average experimental series; they were inseminated according to the methods outlined by Just (1939) and were then washed with freshly drawn, filtered sea water.

The fertilized eggs were allowed to remain undisturbed for a period of 75 minutes after insemination; at the end of this time (shortly before the first cleavage), they were transferred with as little sea water as possible to a series of 25 cc. stender dishes, to which the various solutions of LiCl were then added. Appropriate controls were kept in all series, these being cultured in filtered aerated sea water which was changed at daily intervals. A stock solution of 0.54 M LiCl in distilled water was used for all experimental mixtures; such a solution is approximately isotonic in all dilutions with sea water. Experimental mixtures were designated according to the percentage of this stock solution used—e.g., a "15 per cent solution" indicates that 85 cc. of filtered aerated sea water were added to 15 cc. of the 0.54 M LiCl stock solution. About 15 cc. of the mixture were added to each stender dish; at intervals ranging from 15 minutes to 36 hours, the solutions were decanted carefully and the eggs washed in three or four changes of sea water. LiCl-sea water mixtures ranging from 2 per cent to 100 per cent were tested for varying periods of time. No attempt was made to control the room temperature, which varied from 20° C. to 28° C. However, all cultures were kept in moist chambers which stood in running sea water; the temperature of this sea water averaged about 20° C., and did not vary more than one or two degrees at any time during the entire series of experiments.

Less extensive test series were also conducted for purposes of comparison, using the eggs of *Nereis* from which the vitelline membrane had been removed, according to the method described by Costello (1945a).

¹ The author wishes to express sincere appreciation to Prof. D. P. Costello for advice and encouragement throughout the course of the investigation, to Prof. Viktor Hamburger and Dr. Charles Packard for use of laboratory space at the Marine Biological Laboratory during the summers of 1944 and 1945, to Prof. B. H. Willier for his kindly interest, and to Mr. John S. Spurbeck for valuable assistance with the drawings.

RESULTS

The most striking characteristic of the experimental larvae is the complete absence of exogastrulation, or of any definite evidence of true vegetalization. Abnormal larvae occurred to some extent in almost all the cultures; these abnormalities included the absence of an apical tuft, lengthening of the prototrochal cilia (Fig. 2A), absence of these cilia in varying degrees (Fig. 2), absence or abnormality of the eye spots (Fig. 3), deficiencies in the number of prototrochal cells (Fig. 3), atypical seta sacs with derangements in the number and position of these organs in older larvae (Fig. 4), and abnormalities in the number and position of the oil droplets (Figs. 2C, 3D, 4B). Most of the experimental larvae had abnormalities of the anal and prototrochal pigments; these abnormalities included both the absence of pigment and an abnormal concentration of pigment granules in various regions of the larvae (Figs. 2, 3, 4, 5). The specific occurrence of these anomalies was roughly proportional to the severity of treatment, being far more marked in cultures which

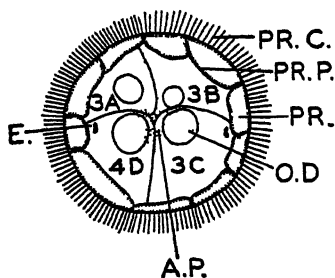


FIGURE 1. A normal *Nereis* trochophore at about 30 hours; polar view. Pr. C. = Prototrochal cilia; Pr. P. = Prototrochal pigment; Pr. = Prototrochal cell; O. D. = Oil droplet; A. P. = Anal pigment; E. = Eye spot; 3A, 3B, 3C, 4D = Entomeres. This and all subsequent text-figures are semi-diagrammatic, based on camera lucida drawings of the living larvae.

were exposed to higher concentrations for sublethal periods. No evidence of twinning was observed.

Complete data describing the results of treatment with the varying concentrations for varying periods are contained in Table I. It is evident that concentrations of 2 per cent to 4 per cent acting for periods up to 12 hours produce larvae which are almost completely normal; similar trochophores result when 5 per cent to 10 per cent solutions are applied for one to 5 hours. However, when allowed to act for periods of 5 to 24 hours, the same concentrations affect the eggs so that the resulting larvae show marked deficiencies in the number of prototrochal cells, and are usually completely devoid of the normal anal and prototrochal pigment. Abnormalities such as these occur quite commonly in all series; some extreme examples are shown in Figure 2. For purposes of comparison, a normal trochophore is shown in Figure 1. Accurate quantitative observations of the exact deficiency in the number of prototrochal cells were impossible in most cases.

Twelve per cent solutions acting for periods of $1\frac{1}{2}$ to 5 hours result in trochophores which are essentially normal, but if allowed to remain on the eggs for 5 to 24 hours, bring about pigment and prototrochal anomalies of the type described above.

TABLE I
Effects of lithium treatment of Nereis eggs

Percentage ^a	Duration of treatment in hours	Appearance of 26-30 hour larvae
2%	1½	Normal
	5	Normal
	8	Mostly normal; some with abnormalities in number and position of oil droplets
	23	Mostly normal; some with abnormalities in number and position of oil droplets
3	8	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	10	Many with incomplete prototroch and with abnormalities in number and position of oil droplets
	24	Abnormal distribution of oil droplets, otherwise normal
4	1½	Normal
	5	Mostly normal
	8	Mostly normal
	12	Normal in form; somewhat sluggish in movements
5	14	Normal
	16	Mostly normal
	18	Mostly normal
	20	Some prototrochal and pigment abnormalities
	22	Pigment and prototrochal abnormalities
	26	Considerable variation in amount of pigment present
	28	Variations in amount of pigment present
	30	More marked pigment abnormalities than after 28-hour treatment
	32	Pigment and prototrochal abnormalities
	36	Dead
6	1½	Normal
	5	Normal, but sluggish in movements
	8	Mostly normal
	12	Normal, but sluggish in movements
	23	Quite abnormal; prototrochal and pigment abnormalities
7	1	Normal
	2	A very few cases with oil droplet deficiencies
	5	Some cases with oil droplet deficiencies
	24	Prototrochal cell deficiencies; sluggish in movements; pigment often absent
8	1½	Normal
	5	Some cases with prototrochal and pigment abnormalities
	8	More marked pigment abnormalities
	12	Severe prototrochal deficiencies; no pigment
	23	Severe prototrochal deficiencies; no pigment. Oil droplets abnormal in number and position. Many larvae without apical tuft

^a Percentages in the table refer to percentages of 0.54 M stock solution of lithium chloride with sea water: 2 per cent = 2 cc. 0.54 M LiCl + 98 cc. sea water.

TABLE I—*Continued*

Percentage ²	Duration of treatment in hours	Appearance of 26–30 hour larvae
10	1	Some localized deposits of pigment; some pigment present in most cases
	4	Some pigmented, some unpigmented. A few cases of abnormality in amount and position of anal pigment
	8	Pigment abnormalities; prototrochal cells seem fairly complete
	12	Pigment abnormalities; prototroch fairly normal
	14	No pigment; marked defects in number of prototrochal cells
	16	No pigment; marked defects in number of prototrochal cells
	18	No pigment, no cilia, no apical tuft
	20	No pigment, no apical tuft. Abnormalities in number and position of oil droplets
	24	No pigment, no cilia, no apical tuft. Abnormalities in number and position of oil droplets
	26	No pigment; marked prototrochal deficiencies. No apical tuft; oil droplets very abnormal in number and position
	28	No pigment; prototrochal defects marked. Oil droplets abnormal in number and position
	36	Dead
12	1½	Fairly normal; some abnormalities in number of prototrochal cells and oil droplets
	5	Many deficiencies of pigment and prototroch; oil droplets abnormal in number and position
	8	Pigment deficiencies; prototroch and oil droplets abnormal in number and position
	12	Some abnormalities in distribution of oil droplets; no pigment. Prototrochal deficiencies; no apical tuft
	23	Dead
14	1½	Mostly normal; some pigment deficiencies
	5	No pigment; marked prototrochal deficiencies
	8	No pigment; prototrochal deficiencies; no apical tuft
	23	Dead
15	1	Mostly unpigmented; prototrochal deficiencies. Anal pigment present in many cases. Oil droplets abnormal in number and position
	2	Unpigmented; prototrochal deficiencies; anal pigment present in some cases. Oil droplet abnormalities
	4	More marked pigment defects; severe prototrochal deficiencies. Some few cases of endodermal extrusion
	6	Highest incidence of endodermal extrusion: 2%–7%
	8	Somewhat fewer cases of extrusion than in 6-hour culture
	10	Some few cases of endoderm extrusion
	12	A very few cases of endodermal extrusion
	14	No pigment; marked prototrochal defects. No apical tuft
	16	No pigment; prototrochal defects; no apical tuft
	20	No cilia, no pigment, no apical tuft. Severe prototrochal deficiencies
	24	No pigment, no cilia, no apical tuft

TABLE I—*Continued*

Percentage ^a	Duration of treatment in hours	Appearance of 26–30 hour larvae
17	1	Anal pigment present; prototrochal pigment absent in many cases. Considerable number of oil droplet abnormalities
	2	No pigment; oil droplet abnormalities; prototrochal deficiencies; no apical tuft
	3	No pigment; oil droplet abnormalities in number and position. Prototrochal deficiencies, no apical tuft
	6	Marked oil droplet deficiencies and abnormalities in position; no pigment. No apical tuft; prototrochal cell deficiencies
	8	Many amorphous forms; no cilia; abnormalities in number and position of oil droplets
	10	No cilia, no pigment, no apical tuft
	14	Dead
20	1	Normal
	2	Pigment abnormalities; some localization of pigment
	4	No pigment; prototrochal cell deficiencies; apical tuft apparently missing; oil droplets abnormal in number and position
	8	Marked oil droplet deficiencies; severe prototrochal defects
	12	Oil droplet abnormalities; marked prototrochal deficiencies
	14	Dead
25	1	Prototrochal cell deficiencies; some pigment defects
	2	No pigment; prototrochal cell deficiencies; abnormalities in number and position of oil droplets
	4	Severe prototrochal deficiencies; no apical tuft. No anal pigment
	8	Prototrochal deficiencies; pigment abnormal. Oil droplet abnormalities in number and position
	12	Marked prototrochal cell deficiencies, no pigment, no apical tuft
	14	Dead
30	2	No pigment; marked prototrochal deficiencies. No apical tuft in most cases
	4	Dead
35	2	No pigment; marked prototrochal deficiencies. No apical tuft
40–90	1	Dead
50	½	No pigment; prototrochal deficiencies. Apical tuft usually absent
100	¼	Prototrochal and pigment deficiencies
	½	Dead

Treatment of the eggs with 15 per cent solutions for one to 4 hours produces pigment and prototrochal abnormalities, but after 6, 8, and 10 hours of treatment, the resulting larvae are marked by peculiar endodermal derangements which were at first thought to be the result of exogastrulation. However, closer examination of these cases revealed that the extruded cytoplasm was non-cellular insofar as could be determined, although it had the characteristic "glassy" appearance of endodermal cytoplasm; usually these larvae were marked by the complete absence of cilia (Fig. 5).

Frequently cases were observed in which the endoderm extended up under the surmounting cap of ectoderm. The cleavage pattern of the experimental eggs of this series showed no abnormality through the fourth cleavage. Repeated experiments with this treatment and with exposure of the eggs to concentrations of 12 per cent and 17 per cent for varying periods of time definitely established the fact that the abnormalities occur only after the treatment described above. It does not seem likely that such an extremely narrow range of dosage would be necessary for the production of true exogastrulae, inasmuch as these forms are produced in sea urchin eggs after a rather wide variety of treatments.

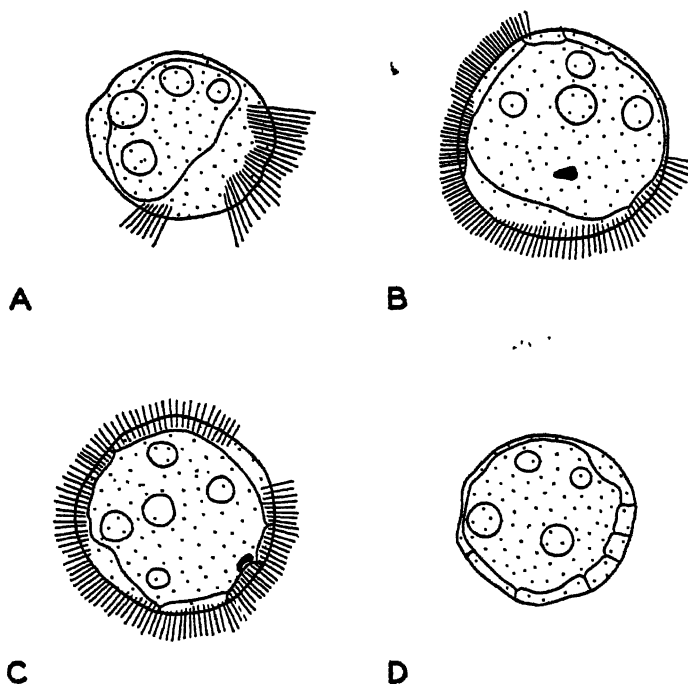


FIGURE 2. Typical 26-30 hour abnormal trochophores, showing anomalies in ciliary band, pigmentation and eye spots. Anal pigment and prototrochal pigment are absent in all cases; Figure 2A shows lengthening of the prototrochal cilia.

Exposure of the eggs to a 17 per cent solution resulted in generally abnormal trochophores after a treatment of one hour, and in more pronounced pigment and prototrochal abnormalities if treated for longer periods, up to the lethal point at 14 hours. No larvae with the endodermal derangements described above were noted in any of the cultures in this series. The effects of a 20 per cent solution for comparable periods of time approximate those described for a 17 per cent treatment; a concentration of 25 per cent produces pigment and prototrochal abnormalities after treatment for 1, 2, 4 and 8 hours. These defects also result from treatment for 2 hours with a 30 per cent solution. Concentrations of 40 per cent to 90 per cent

are lethal after treatment for one hour, and a 100 per cent solution has the same effect after $\frac{1}{2}$ hour; the 100 per cent concentration produces pigment and prototrochal defects if applied for 15 minutes, so that its range of dosage is apparently very narrow. The reduction of the prototroch seems to increase quantitatively with an increase in the concentration of lithium and the duration of application.

Denuded Nereis eggs proved to be extremely sensitive to the effects of lithium, even in low concentrations for short periods of time. In all series, the controls showed considerable abnormality in shape and in the distribution of pigment, so that

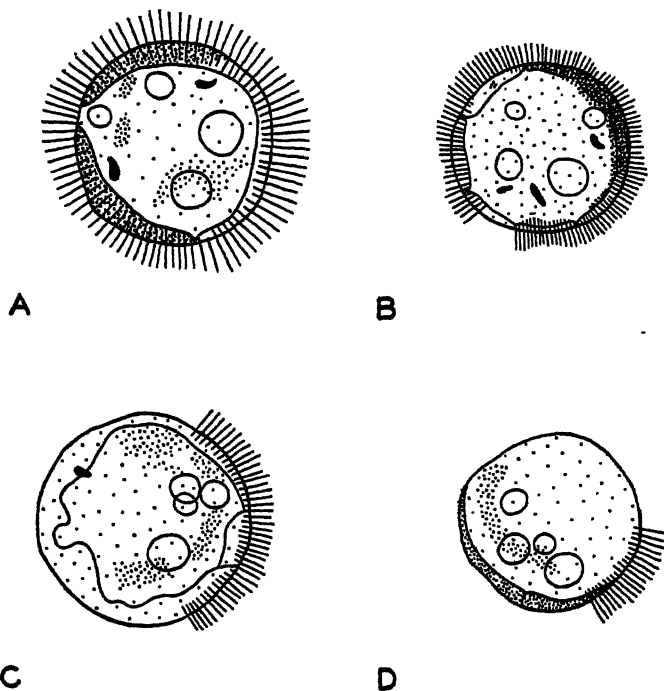


FIGURE 3. Abnormalities involving pigment, eye spots, cilia, and oil droplets after various treatments with lithium. Figure 3A is almost normal except for the heavy concentrations of anal and prototrochal pigment; Figure 3B indicates an abnormality in the number and position of the eye spots and in the size of these structures.

it is difficult to come to any definite decision as to the specific effects brought about by the lithium. However, in the cases in which membrane removal was not complete, the larvae survived fairly well, and in none of these cases were any indications of exogastrulation observed. After treatment for 2 hours with 15 per cent LiCl , some of the completely denuded larvae seemed to show signs of exogastrulation, although no conclusions can be drawn from this because of the small number of cases. Treatment of the denuded eggs with 5 per cent solutions for periods longer than 4 hours killed the eggs in late stages of cleavage, and no definite effects could be noted in larvae surviving in cultures which had been exposed for periods of 1 and 2 hours.

DISCUSSION

From the foregoing results, it is apparent that the main effect of the lithium is to produce a reduction in the number of prototrochal cells, and in the quantity of anal and prototrochal pigments, together with effects on the eye spots and cilia. The absence of the apical tuft in most cases of prolonged or concentrated treatment indicates that the 1a-1d cells may be affected directly or indirectly, since it is known from the observations of Wilson (1892) that this quartet is associated with the formation

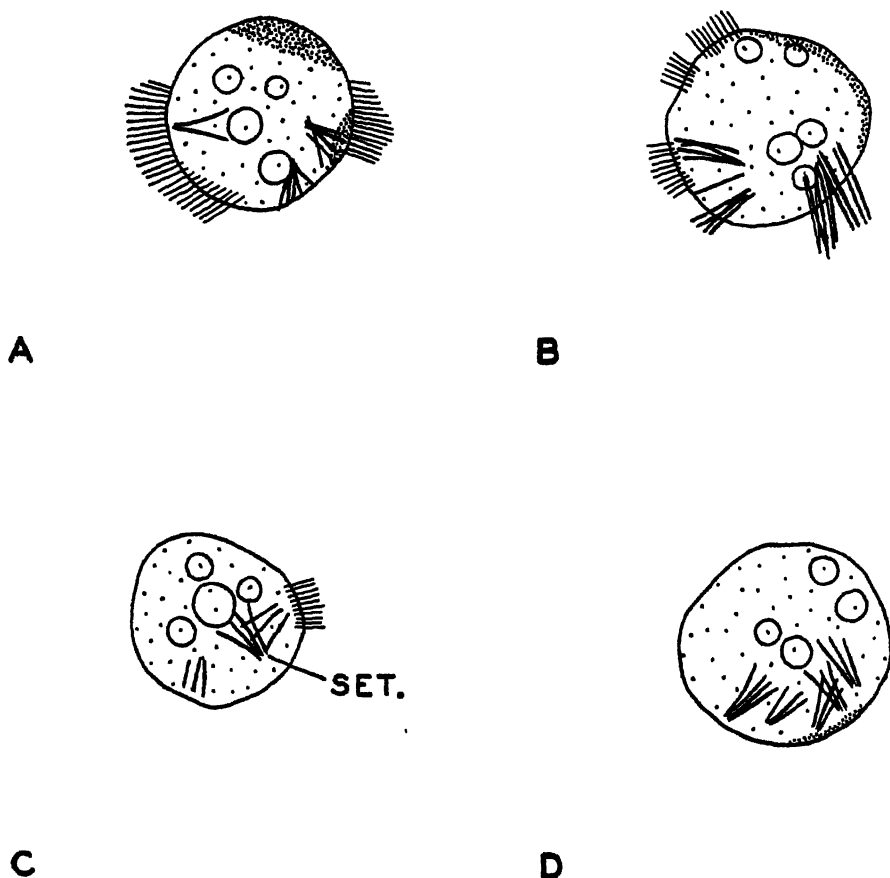


FIGURE 4. Marked abnormalities in setae of older larvae (ca. 53 hours). Figure 4B also shows an atypical number of oil droplets. Set. = Setae.

of the apical tuft. It is possible that the abnormalities observed in the seta sacs of older larvae (Fig. 4) may be due to the early action of the ion on the material destined to be incorporated into the 2d cell.

The absence of any true cases of exogastrulation in these experiments is quite striking. As was noted above, most of the forms in which this abnormality has been observed are marked by a type of gastrulation in which invagination plays a main

role. In the amphibian, formation of the endoderm is accomplished by a complex series of cell movements, as a result of which a directed and organized migration of cells brings about invagination of most of the vegetal hemisphere at the region of the blastopore. Meanwhile, the animal cells grow down to cover the vegetal region, thus forming the ectoderm of the embryo. The yolk material itself is invaginated in these forms and comes to be enclosed by the endoderm. Gastrulation in the echinoderms is likewise thought by Herbst (1893) to be brought about by an invaginative process. The strictly epibolic form of gastrulation exhibited by the egg of *Nereis*, on the other hand, is accomplished by a downgrowth of the ectomeres, so that even-

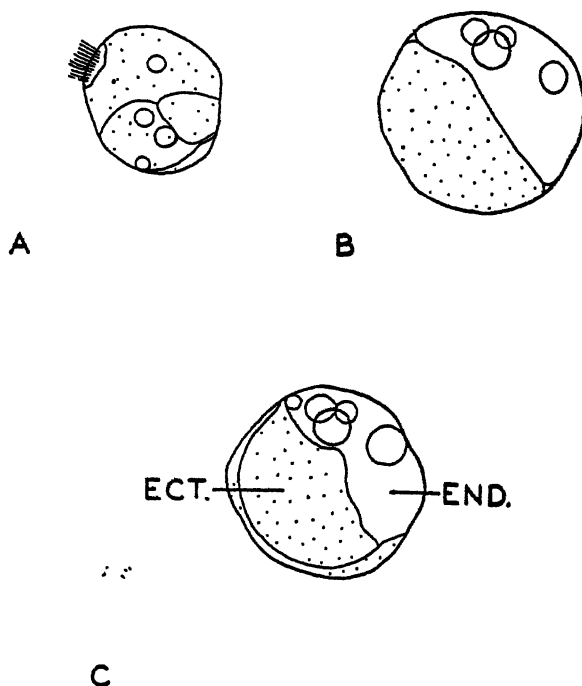


FIGURE 5. Abnormal trochophores resulting from treatment of eggs with 15 per cent LiCl for 6, 8 and 10 hours. Figure 5A shows no particular endodermal derangements, but has only one small tuft of cilia and is quite atypical in shape. Figures 5B and 5C show examples of "endodermal extrusions." Cell boundaries were not visible except as indicated. Ect. = Ectoderm; End. = Endoderm.

tually they cover the four entomeres. Such a movement seems to be associated with the presence of a "cellular affinity" as postulated by Costello (1940, 1945a) in connection with his experiments on the development of isolated blastomeres of this egg. Since no exogastrulation occurred, it may be assumed that this dynamic association of ectomeres and entomeres is not radically disturbed. However, the abnormalities in the number and position of the oil droplets in the four entomeres indicate that the lithium may exert some effect on the process of cytoplasmic segregation (Costello, 1945b) preceding the formation of the 3A, 3B, 3C and 4D cells.

No distinct line of demarcation can be drawn between the direct and the indirect effects of lithium in these experiments, since the ion is brought into contact with the egg before cleavage has occurred, and, in the case of the longer exposures, may remain until considerably after the completion of gastrulation. Runnström (1928b), observing lithium-treated sea urchin eggs under dark-field illumination, presented evidence that the element actually penetrates the cells. Spek (1918) maintained that the action of the lithium ion was brought about through its production of a precipitation and swelling effect on the surface of the vegetative cells. Thus, the exact mode of action remains obscure, but it appears fairly clear in the case of the egg of *Nereis* that exogastrulation is not produced, at least not in cases where the vitelline membrane is present.

The effects produced on the pigment of the trochophores seem to be at random, since the anal pigment may occur without the presence of the prototrochal pigment, and vice versa—or both may be present in varying degrees. This action possibly is related to the orientation of the eggs with respect to the bottom of the dish, or to each other; although no particular effort was made to keep the eggs suspended in the solution, the jelly serves to support them during the early stages of development, thus allowing relatively free access of the lithium to all surfaces.

SUMMARY

1. The fertilized eggs of *Nereis limbata* were treated with mixtures of sea water and a 0.54 M stock solution of LiCl, ranging from 2 per cent to 100 per cent for periods of 15 minutes to 36 hours. Treatment was begun 75 minutes after insemination of the eggs, shortly before the appearance of the first cleavage.

2. No exogastrulae were observed. There were a few cases of marked abnormalities in the endodermal components of the trochophores within a very narrow range of treatment (15 per cent for 6, 8 and 10 hours).

3. The main abnormalities observed in the experimental larvae were: Absence of the apical tuft, lengthening of the prototrochal cilia, absence of these cilia in varying degrees, abnormalities in the anal and prototrochal pigments, absence or abnormality of the eye spots, deficiencies in the number of prototrochal cells, atypical seta sacs, abnormalities in the number and position of oil droplets. The degree of abnormality in these cases seemed to be roughly proportional to the severity of treatment.

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE
 CHATTON AND LWOFF (ORDER HOLOTRICHA, SUBORDER
 THIGMOTRICHA). II. HYPOCOMIDES MYTILI CHATTON
 AND LWOFF, HYPOCOMIDES BOTULAE SP. NOV.,
 HYPOCOMIDES PARVA SP. NOV., HYPOCOMIDES
 KELLIAE SP. NOV., AND INSIGNICOMA
 VENUSTA GEN. NOV., SP. NOV.

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INTRODUCTION

The genus *Hypocomides* was proposed by Chatton and Lwoff (1922a) to include two species: *Hypocomides mytili*, from *Mytilus edulis* L., and *H. modiolariae*, from *Modiolaria marmorata* (Forbes). These authors gave no formal diagnosis of the genus *Hypocomides*, however, and their incomplete descriptions of the two species are not supplemented by illustrations. They did not designate either species as the genotype. In 1926 Chatton and Lwoff published a preliminary diagnosis, again without illustrations, of a third species of *Hypocomides*, *H. syrphacae*, from *Zirfaea crispata* (L.). It was largely on the basis of their description of *H. syrphacae* that Raabe (1938) was led to suppose that one of the ciliates which he found parasitizing the gills of *Mytilus edulis* was *H. mytili*. He could not identify on this ciliate, however, a structure to which Chatton and Lwoff referred as the "vestige de frange adorale" and which according to them is in *H. mytili* "constitué seulement par une dizaine de grands cils." This "vestige de frange adorale" is supposed to be better developed in *H. modiolariae* than in *H. mytili*. In *H. syrphacae* it is represented, according to Chatton and Lwoff, by "une touffe de cils." The position of the "vestige de frange adorale" in relation to other ciliary structures in these three species of *Hypocomides* is entirely unclear.

On a ciliate which I have studied from *Mytilus edulis* from San Francisco Bay and which conforms in most respects to the description given by Raabe of the form considered by him to be *H. mytili*, I have been unable to detect such a "vestige de frange adorale." The brief notes on the morphology of *H. mytili* to be found in the papers of Chatton and Lwoff in which mention is made of this species (1922a, 1922b, 1924) are not entirely consistent, and it is altogether possible that, as Raabe has pointed out, these authors used the term "vestige de frange adorale" to indicate only a short segment of the distal portion of one of the longer ciliary rows on the right side of the body, which Raabe suggested may be homologous with the two rows bordering the peristomal groove of ciliates of the family Ancistrumidae. It is quite evident from the brief description of *H. syrphacae*, however, that the "vestige de frange adorale" of this species is entirely separate from the two long rows on the right. Perhaps *H. mytili* and *H. modiolariae* are not actually congeneric with *H. syrphacae*, but this remains to be seen. At any rate, unless it can be established

with certainty that the ciliate described by Chatton and Lwoff as *Hypocomides mytili* is not identical with the species thought by Raabe to be *H. mytili*, it seems best to consider the form studied by Raabe to be *H. mytili* and to refer related forms to the same genus.

In the present paper I will give a description of the ciliate from *Mytilus edulis* which I consider to be *H. mytili*, and will add three new species to the genus *Hypocomides*: *H. botulae* sp. nov. and *H. parva* sp. nov., from the gills and palps of the rock-boring pelecypod, *Botula californiensis* (Philippi), and *H. kelliae* sp. nov., from *Kellia laperousii* Deshayes, a small nestling clam which is frequently encountered in the excavations made in rocks by other molluscs. Another very interesting ancistrocomid ciliate from *Botula californiensis* will be described herein as *Insigni-coma venusta* gen. nov., sp. nov.

HYPOCOMIDES MYTILI CHATTON AND LWOFF

(Fig. 1; Plate I, Fig. 1)

The body is elongated and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior three-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually not quite so rounded as the right margin, and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Thirty living individuals taken at random ranged in length from $34\ \mu$ to $48\ \mu$, in width from $16\ \mu$ to $22\ \mu$, and in thickness from $13\ \mu$ to $18\ \mu$, averaging about $40\ \mu$ by $18\ \mu$ by $14.5\ \mu$.

The anterior end of the body is provided with a short contractile tentacle which enables the ciliate to attach itself to the epithelial cells of the gills and palps of the host and to suck out their contents. This tentacle is continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for about one-half the length of the body. The canal nearly always appears to be widest in its anterior portion and to be directed obliquely toward the right side of the body as it extends posteriorly.

The cilia of *H. mytili* are about $9\ \mu$ in length and are markedly thigmotactic, especially near the anterior end of the body. The ciliary system consists of three separate complexes. The central complex, occupying the middle and right portions of the ventral anterior concavity, consists of seven rows, the one nearest the right being the shortest (one-third to two-fifths the length of the body), the other rows becoming progressively longer toward the left. The sixth and seventh rows are usually approximately one-half the length of the body; in some specimens the seventh row is appreciably shorter than the sixth. To the right of the central complex are two long rows, each about one-half the length of the body. Both these rows originate on the dorsal surface close to the left margin a short distance behind the level of origin of the rows of the central complex and curve ventrally as they extend posteriorly. To the left of the central complex is a series of eight rows which usually are more closely-set than those making up the central complex. The innermost row, which originates on the left margin of the body near the base of the suckorial tentacle, is the shortest, and terminates at a point about one-third the distance from the anterior end of the body to the posterior end. The remaining rows become pro-

gressively longer and originate progressively more dorsally and posteriorly, curving ventrally as they extend backward. The outer row of this complex is usually the longest and terminates at a point nearly opposite the point of termination of the outer of the two rows constituting the complex on the right.

According to Raabe, the three ciliary complexes of *H. mytili* are much more distinctly separated than I have observed them to be. Raabe also stated that variations in the number of ciliary rows in the central and left complexes are correlated with two well-differentiated size races: form *minor* ($17\ \mu$ to $26\ \mu$ in length) and form *major* ($26\ \mu$ to $36\ \mu$ in length). I have noted no tendency for the ciliates I consider to belong to this species to be segregated into distinct size races and have seen no examples of *H. mytili* which were in life as small as those assigned to the form *minor* by Raabe. Although I have observed few deviations from the typical number of ciliary rows, it may be of interest to record here the fact that frequently some

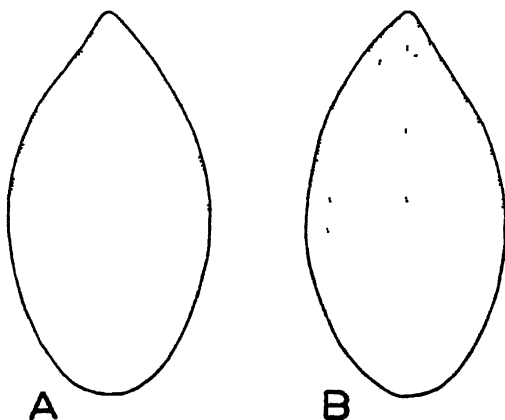


FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Distribution of ciliary rows. somewhat diagrammatic.¹ A, dorsal aspect; B, ventral aspect.

of the rows of the left complex, particularly the first and second rows, do not stain as well with hematoxylin or impregnate as well with activated silver albumose (protargol) as do the rows of the central and right complexes, and hence may easily escape detection.

The cytoplasm is colorless and contains numerous lipid droplets in addition to food inclusions. Several larger food vacuoles are sometimes observed in the posterior part of the body. The contractile vacuole is centrally located² and opens to the exterior on the ventral surface. I have observed no permanent opening in the pellicle.

The macronucleus is usually ovoid, sometimes sausage-shaped; more rarely it is spherical, although Raabe considered the macronucleus of *H. mytili* to be typically

¹ All text figures illustrating this paper are based on camera lucida drawings of individuals fixed in Schaudinn's fluid and impregnated with activated protein silver (protargol).

² Raabe stated that the contractile vacuole of *H. mytili* is to be found in a vacuolated area in the posterior part of the body behind the macronucleus. Perhaps his observation was based on specimens which were undergoing degenerative vacuolization and in which the contractile vacuole was not conspicuous. In all normal individuals which I have examined its position was central.

spherical. It is situated dorsally in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen nuclear reaction the macronucleus ranged in length from $9\ \mu$ to $13.2\ \mu$ and in width from $4.4\ \mu$ to $6.9\ \mu$.

The spherical micronucleus is ordinarily situated near the middle of the body anterior to the macronucleus, although sometimes it is seen to lie to one side of the macronucleus. The chromatin appears in most fixed and stained specimens to be homogeneous, although in some it is aggregated into vague peripheral granules or strands. In fifteen individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from $2.7\ \mu$ to $4\ \mu$.

I found *Hypocomides mytili* to be present in large numbers on the gills and palps of about 80 per cent of the specimens of *Mytilus edulis* which I examined from various localities in San Francisco Bay. It is sometimes the only ciliate infesting the mussels, but more commonly it is associated with *Crebricoma carinata* (Raabe) and *Ancistruma mytili* (Quennerstedt).

Hypocomides mytili Chatton and Lwoff

Diagnosis: Length $34\ \mu$ – $48\ \mu$ (according to Raabe $17\ \mu$ to $36\ \mu$), average about $40\ \mu$; width $16\ \mu$ – $22\ \mu$, average about $18\ \mu$; thickness $13\ \mu$ – $18\ \mu$, average about $14.5\ \mu$. The central ciliary complex is composed of seven rows (according to Raabe seven or eight rows) which are one-third to one-half the length of the body, becoming progressively longer toward the left side; the right complex consists of two rows, each about one-half the length of the body; the left complex consists of eight closely-set rows (according to Raabe five [?] or six rows) one-third to one-half the length of the body. The contractile vacuole is central and opens to the exterior on the ventral surface. The macronucleus is typically ovoid or sausage-shaped, rarely spherical. The micronucleus is spherical. Parasitic on the epithelium of the gills and palps of *Mytilus edulis* L. (Roscoff [Chatton and Lwoff]; Hel [Raabe]; San Francisco Bay, California).

HYPOCOMIDES BOTULAE SP. NOV.

(Fig. 2; Plate 1, Fig. 2)

The body is elongated, narrowed anteriorly, and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-half of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior portion of the left margin is usually less rounded than the right margin and appears typically to be weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from $31\ \mu$ to $39\ \mu$, in width from $14\ \mu$ to $17\ \mu$, and in thickness from $12\ \mu$ to $14\ \mu$, averaging about $33\ \mu$ by $15\ \mu$ by $13\ \mu$.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal which can usually be traced in fixed specimens stained with iron hematoxylin for a distance equal to about three-fifths the length of the body.

In most individuals the canal appears to be directed obliquely toward the right side of the body.

The cilia are about 8μ to 9μ in length and are markedly thigmotactic, particularly near the base of the suctorial tentacle. The ciliary system consists of three separate complexes. The central complex, occupying the larger part of the ventral anterior concavity, consists of eleven rows about one-half the length of the body which become progressively longer toward the left side. To the right of the central complex are two longer rows which originate on the dorsal surface close to the right margin a little behind the level of origin of the rows of the central complex and curve downward as they extend posteriorly. Each of the two rows is about three-fifths the length of the body. To the left of the central complex is a series of eleven rather closely-set rows which originate on the left lateral margin and the dorsal surface on the left side and curve ventrally as they extend posteriorly. These

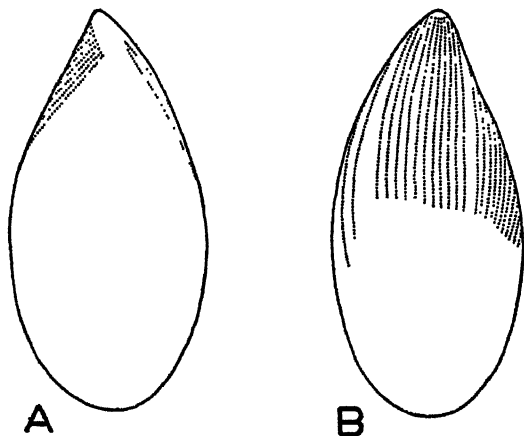


FIGURE 2. *Hypocomides botulac* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

rows are about one-half the length of the body and originate and terminate progressively more posteriorly.

The cytoplasm is colorless and contains numerous lipid droplets in addition to food inclusions. I have not seen any large food vacuoles in this species. The contractile vacuole, situated near the middle of the body, opens to the exterior on the ventral surface.

The macronucleus is ovoid to sausage-shaped and is situated in the posterior half of the body. Its longitudinal axis is usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin is aggregated into a dense reticulum. Vacuole-like clear spaces of varying size are sometimes apparent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 9μ to 13μ and in width from 4.3μ to 7μ .

The micronucleus is spherical and is situated dorsally near the middle of the body. The chromatin appears to be homogeneous in fixed specimens. In ten individuals

fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from $2.4\ \mu$ to $3.2\ \mu$.

Hypocomides botulae was present in small numbers on the gills and palps of twelve of the thirty-four specimens of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes found in association with *Hypocomides parva* and *Insignicoma venusta*. No specimens of *Botula falcata* (Gould) which I examined from the same localities were parasitized by these or any other ciliates.

Hypocomides botulae sp. nov.

Diagnosis: Length $31\ \mu$ – $39\ \mu$, average about $33\ \mu$; width $14\ \mu$ – $17\ \mu$, average about $15\ \mu$; thickness $12\ \mu$ – $14\ \mu$, average about $13\ \mu$. The central ciliary complex consists of eleven rows each about one-half the length of the body; the right complex is composed of two rows about three-fifths the length of the body; the left complex consists of eleven closely-set rows about one-half the length of the body. The macronucleus is ovoid to sausage-shaped. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMIDES PARVA SP. NOV.

(Fig. 3; Plate I, Fig. 3)

The body is elongated, narrowed anteriorly, and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior two-fifths of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin, and appears typically to be nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty-five living individuals taken at random ranged in length from $21\ \mu$ to $29\ \mu$, in width from $10\ \mu$ to $13\ \mu$, and in thickness from $8\ \mu$ to $11\ \mu$, averaging about $26\ \mu$ by $12\ \mu$ by $10\ \mu$.

The anterior end of the body is provided with a short contractile suckorial tentacle continuous with an internal tubular canal. The canal is usually directed obliquely toward the right side and can be traced in most fixed specimens stained with iron hematoxylin for about one-half the length of the body.

The cilia of *Hypocomides parva* are about $6\ \mu$ to $7\ \mu$ long and are strongly thigmotactic, particularly near the base of the suckorial tentacle. The ciliary system consists of three separate complexes. The central complex, which occupies the larger part of the concave depression on the anterior part of the ventral surface, consists of eight rows which are about two-fifths the length of the body. To the right of this complex are two longer rows which originate on the dorsal surface close to the right margin behind the level of origin of the rows that constitute the central complex. Each of these rows is about three-fifths the length of the body and curves ventrally as it extends posteriorly. The outer row originates and terminates the more posteriorly. To the left of the central complex is a series of eight rows which originate on the left lateral margin and on the left side of the dorsal surface and curve ventrally as they extend backward. These rows are about two-fifths the length of the body and originate and terminate progressively more posteriorly. The outermost

row, however, is somewhat shorter than the other rows and is usually seen to terminate a little anterior to the point of termination of the seventh row.

The cytoplasm is colorless and contains numerous small lipid droplets and food inclusions. I have distinguished no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is typically ovoid, sometimes spherical, rarely sausage-shaped. It is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing a few vacuole-like clear spaces of varying size. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from $4.2\ \mu$ to $8.2\ \mu$ and in width from $4.2\ \mu$ to $5.3\ \mu$.

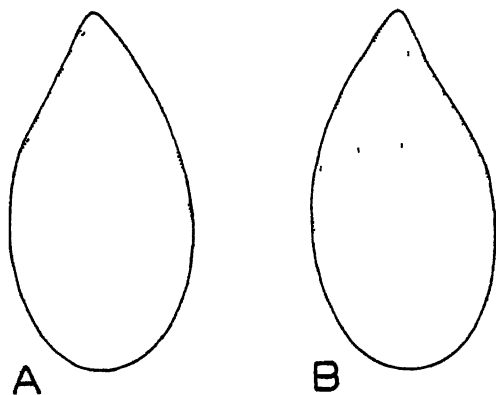


FIGURE 3. *Hypocomides parva* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

The micronucleus is usually spherical and is situated dorsally a short distance anterior to or to one side of the macronucleus. Sometimes it is located as far anteriorly as the middle of the body. In fixed and stained preparations the chromatin of the micronucleus appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from $1.9\ \mu$ to $2.3\ \mu$.

Hypocomides parva was present on the gills and palps of nineteen of the thirty-four individuals of *Botula californiensis* which I examined from localities near Moss Beach, California. It is sometimes associated with *H. botulae* and *Insignicoma venusta*. In my experience it is the most common species of ciliate parasitizing this mollusc.

Hypocomides parva sp. nov.

Diagnosis: Length $21\ \mu$ – $29\ \mu$, average about $26\ \mu$; width $10\ \mu$ – $13\ \mu$, average about $12\ \mu$; thickness $8\ \mu$ – $11\ \mu$, average about $10\ \mu$. The central ciliary complex comprises eight approximately equal rows about two-fifths the length of the body;

the right complex consists of two rows about three-fifths the length of the body; the left complex consists of eight rows, each about two-fifths the length of the body, the eighth row being usually somewhat shorter than the others. The macronucleus is typically ovoid. The micronucleus is spherical. Parasitic on the gills and palps of *Botula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMIDES KELLIAE SP. NOV.

(Fig. 4; Plate I, Fig. 4)

The body is elongated, narrowed anteriorly, and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the shallow concavity occupying the anterior one-third of the ventral sur-

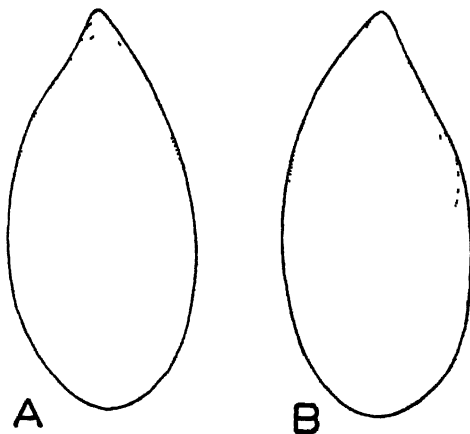


FIGURE 4. *Hypocomides kelliae* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

face; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is not as rounded as the right margin, and typically is nearly straight or weakly indented. The body is widest near the middle and rounded posteriorly. Twenty living individuals taken at random ranged in length from $31\ \mu$ to $37\ \mu$, in width from $13\ \mu$ to $15\ \mu$, and in thickness from $11\ \mu$ to $13\ \mu$, averaging about $33\ \mu$ by $14\ \mu$ by $12\ \mu$.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The canal is usually directed toward the right side of the body as it extends posteriorly. It can be traced in most fixed individuals stained with iron hematoxylin for about three-fifths the length of the body.

The thigmotactic cilia of *H. kelliae* are about $8\ \mu$ or $9\ \mu$ in length. The ciliary system is composed of three separate complexes. The central complex, occupying the larger part of the anterior ventral depression, consists of five equal rows about one-third the length of the body. To the right of this system is a single long row about two-thirds the length of the body. This row originates on the dorsal surface

close to the right margin a little behind the level of origin of the central rows and curves ventrally as it extends posteriorly. To the left of the central complex is a series of five rows, one-third the length of the body, which originate on the left lateral margin or dorsal surface on the left side and curve ventrally as they extend posteriorly. These rows originate and terminate progressively more posteriorly.

The cytoplasm is colorless and contains numerous small lipid droplets and food inclusions. I have observed no large food vacuoles in this species. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped, usually about two times as long as wide. It is situated in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be organized into a very dense reticulum which sometimes is seen to enclose vacuole-like clear spaces of varying size. These are more evident near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from $7.8\ \mu$ to $14\ \mu$ and in width from $3.9\ \mu$ to $7\ \mu$.

The micronucleus varies in shape from spherical to ovoid; typically it is ovoid. It is usually situated dorsally a short distance anterior to the middle of the body or to one side of the anterior part of the macronucleus. In most fixed and stained individuals of *H. kelliae* the chromatin of the micronucleus is aggregated into deeply-staining peripheral strands. In ten specimens fixed in Schaudinn's fluid and stained by the Feulgen reaction the micronucleus ranged in size from $1.9\ \mu$ by $1.5\ \mu$ to $2.3\ \mu$ by $1.9\ \mu$.

Hypocomides kelliae was present in nine of the twenty-eight individuals of *Kellia laperousii* which I examined from localities near Moss Beach, California. Also associated with this mollusc is a small ancistriumid ciliate which it may be possible for me to describe in a subsequent paper.

Hypocomides kelliae sp. nov.

Diagnosis: Length $31\ \mu$ – $37\ \mu$, average about $33\ \mu$; width $13\ \mu$ – $15\ \mu$, average about $14\ \mu$; thickness $11\ \mu$ – $13\ \mu$, average about $12\ \mu$. The central ciliary complex consists of five rows about one-third the length of the body; the right complex consists of a single row about two-thirds the length of the body; the left complex consists of five rows about one-third the length of the body. The macronucleus is ovoid or sausage-shaped. The micronucleus is typically ovoid. Parasitic on the epithelium of the gills and palps of *Kellia laperousii* Deshayes (Moss Beach, California). Syntypes are in the collection of the author.

INSIGNICOMA VENUSTA GEN. NOV., SP. NOV.

(Fig. 5; Plate I, Fig. 5)

The body is elongated, narrowed anteriorly, and somewhat flattened dorso-ventrally. The anterior one-half of the ventral surface, on which the major part of the ciliary system is disposed, is weakly concave; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The anterior half of the left margin is usually not so rounded as the right margin and typically is nearly straight or slightly indented. The body is widest near the middle and rounded poste-

riorly. Twenty-five living individuals taken at random ranged in length from $42\ \mu$ to $52\ \mu$, in width from $18\ \mu$ to $21\ \mu$, and in thickness from $15\ \mu$ to $18\ \mu$, averaging about $48\ \mu$ by $20\ \mu$ by $17\ \mu$.

The anterior end of the body is provided with a contractile suctorial tentacle continuous with an internal tubular canal. In most fixed specimens stained with iron hematoxylin the canal can be traced for about three-fifths the length of the body. It usually appears to be directed obliquely toward the right side.

The ciliary system consists of four separate complexes. Two long, widely-spaced rows on the right side of the body originate on the dorsal surface close to the right margin at the anterior end and are about two-thirds the length of the body. They curve ventrally as they extend posteriorly. A central complex of fourteen or fifteen

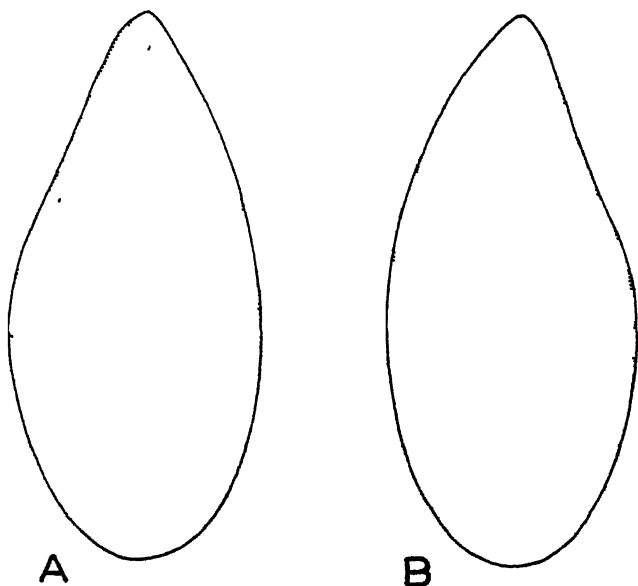


FIGURE 5. *Insignicola venusta* gen. nov., sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

rows occupies the larger part of the ventral anterior depression. These rows are on the average about one-half the length of the body and originate progressively more posteriorly toward the left side. The outer two or three rows on the left, however, do not usually terminate quite so far posteriorly as the twelfth row. The rows of this complex are usually a little more closely-set on the right side than on the left. To the left of the central complex is a series of sixteen or seventeen rows about one-half the length of the body which with the exception of the outer three or four rows are very closely-set. The innermost row originates on the left lateral margin near the base of the suctorial tentacle; the remaining rows originate progressively more dorsally and posteriorly. The distal portions of several of the inner rows of this complex are usually visible on the left side of the ventral surface. Posterior to the middle of the body on the left side is a nearly V-shaped series of cilia which originates

on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of this fourth complex are about $12\ \mu$ to $14\ \mu$ in length. The cilia of the other rows are about $8\ \mu$ or $9\ \mu$ in length and strongly thigmotactic, especially near the base of the suctorial tentacle.

The cytoplasm is colorless and contains numerous small lipoid droplets in addition to food inclusions. A few larger food vacuoles are sometimes observed near the posterior end. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid or sausage-shaped and is situated in the posterior half of the body with its longitudinal axis usually placed obliquely to the longitudinal axis of the body. In fixed and stained preparations the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying sizes. These are most prominent near the periphery. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from $12\ \mu$ to $17\ \mu$ and in width from $4.4\ \mu$ to $9\ \mu$.

The spherical micronucleus is commonly situated dorsally a short distance anterior to or to one side of the macronucleus. In fixed and stained preparations the chromatin appears to be homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from $2.4\ \mu$ to $4\ \mu$.

Insignicoma venusta was found to parasitize the gills and palps of nine of the thirty-four specimens of *Botula californiensis* which I collected at localities near Moss Beach, California.

Insignicoma gen. nov.

Diagnosis: The body is elongated and somewhat flattened dorso-ventrally. The anterior end of the body is narrowed and provided with a contractile suctorial tentacle continuous with an internal tubular canal. The ciliary system consists of four separate complexes. The central complex, occupying the major portion of the shallow concavity on the anterior one-half of the ventral surface, is bounded on the right by a small number of widely-spaced rows which curve ventrally as they extend posteriorly; to the left of the central complex is a series of closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left lateral margin and dorsal surface on the left side and curve ventrally as they extend backward; the fourth complex consists of a V-shaped series of long cilia which lies immediately behind the distal portion of the gap separating the central and left ciliary complexes. The contractile vacuole is central and opens to the exterior

PLATE I

All figures have been drawn with the aid of a camera lucida from specimens fixed in Schaudinn's fluid and stained with iron hematoxylin. $\times 1870$.

FIGURE 1. *Hypocomides mytili* Chatton and Lwoff. Ventral aspect.

FIGURE 2. *Hypocomides botulacae* sp. nov. Ventral aspect.

FIGURE 3. *Hypocomides parva* sp. nov. Ventral aspect.

FIGURE 4. *Hypocomides kelliae* sp. nov. Ventral aspect.

FIGURE 5. *Insignicoma venusta* gen. nov., sp. nov. Ventral aspect.

PLATE I



on the ventral surface; there is no permanent opening in the pellicle. Genotype: *Insignicomu venusta* gen. nov., sp. nov.

Insignicomu venusta gen. nov., sp. nov.

Diagnosis: Length $42\text{ }\mu\text{--}52\text{ }\mu$, average about $48\text{ }\mu$; width $18\text{ }\mu\text{--}21\text{ }\mu$, average about $20\text{ }\mu$; thickness $15\text{ }\mu\text{--}18\text{ }\mu$, average about $17\text{ }\mu$. The central ciliary complex consists of fifteen (rarely fourteen) rows about one-half the length of the body which originate progressively more posteriorly toward the left side; the right complex consists of two widely-spaced rows about two-thirds the length of the body which originate on the dorsal surface close to the left margin and curve ventrally as they extend posteriorly; the left complex consists of sixteen or seventeen closely-set rows about one-half the length of the body which originate progressively more posteriorly on the left margin and dorsal surface on the left side and curve ventrally as they extend posteriorly; the V-shaped series of cilia constituting the fourth complex originates on the ventral surface in the posterior third of the body, extends anteriorly and to the left to a point a short distance behind the distal portion of the gap separating the central and left ciliary complexes, then bends abruptly backward and dorsally. The cilia of the fourth complex are approximately $12\text{ }\mu\text{--}14\text{ }\mu$ in length; those of the other three complexes are approximately $8\text{ }\mu\text{--}9\text{ }\mu$ in length. The macronucleus is ovoid or sausage-shaped; the micronucleus is spherical. Parasitic on the gills and palps of *Rotula californiensis* (Philippi) (Moss Beach, California). Syntypes are in the collection of the author.

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NATURAL HETEROAGGLUTININS IN THE BODY-FLUIDS AND SEMINAL FLUIDS OF VARIOUS INVERTEBRATES¹

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INTRODUCTION

Since the early work of Landois (1875) numerous investigators have noted in the normal serum of various species of animals, particularly among the vertebrates, the occurrence of agglutinins that act on the cells of certain other species (cf. Wiener, 1943; Landsteiner, 1945; Thomsen, 1932). Such natural heteroagglutinins have also been frequently reported to occur in the serum or body-fluids of various invertebrates (see literature in Huff, 1940; Tyler and Metz, 1945). Heteroagglutination reactions are frequently encountered in fertilizin studies and have been reported by several investigators (Lillie, 1913, 1919; Glaser, 1914; Just, 1919, 1930; Sampson, 1922; Godlewski, 1934; Hartmann *et al.*, 1940; Runnström *et al.*, 1944) as occurring between spermatozoa and foreign egg-water preparations, body-fluids and spermatozoa or their extracts. Further information concerning the range and nature of the heteroagglutination reactions is of importance, then, in analysis of problems of fertilization particularly in regard to the specificity and role of the interacting substances that are obtained from eggs and sperm.

A study of heteroagglutination reactions with lobster serum (Tyler and Metz, 1945; Tyler and Scheer, 1945), which normally acts on the sperm or the blood-cells of a wide variety of species throughout the animal kingdom, showed that at least ten distinct, relatively class-specific agglutinins are present in the serum of this species. This was determined by means of absorption tests. In the controls for those tests the supernatant fluids of the sperm suspensions used for absorption were also examined for agglutinating activity and it was found, particularly when the sperm had not been previously washed, that the fluids from sperm of some species were active on cells of certain other species. Tests were then made with the body-fluids and these, too, were found to be active.

The present paper reports the results of this examination of the body-fluids and the sperm-supernatants of various species of invertebrates for the occurrence of heteroagglutinins. The species examined were for the most part those that have been used or are potentially useful in fertilizin studies. In addition a few absorption tests were made with starfish body-fluid to determine whether or not its agglutinating activity is attributable to the presence of several heteroagglutinins, each with broad specificity such as was found in lobster serum.

MATERIALS AND METHODS

The body-fluids of twelve species of animals among the annelids, echinoderms, mollusks and tunicates were examined for possible agglutinating action on sperm

¹ This work has been aided by a grant from the Rockefeller Foundation. I am indebted to Miss Margaret L. Campbell for technical assistance.

suspensions of various species of invertebrates and, in a few cases, on erythrocyte suspensions of various vertebrates. The body-fluids were obtained by incision into the body cavity or by insertion of a hypodermic syringe into the body cavity. In the case of *Ciona* the fluid was obtained directly from the heart. The fluids were clarified by centrifugation and tested in the manner previously described (Tyler and Metz, 1945). The supernatant fluids of sperm suspensions of eleven of the same species were also tested. These were obtained by centrifugation of approximately 10 to 20 per cent suspensions of "dry" sperm in sea water. They may be termed diluted seminal fluids.

EXPERIMENTAL PART

In all of the species that were examined the body-fluids were found to possess agglutinating activity for the cells of certain other species. The results are presented in Table I. In some species (e.g., 1, 6, 18, 19, 21) the fluids exhibited agglutinating action on the cells of most of the species that were tested. However, the fluids of

TABLE I

Heteroagglutinating action of body-fluid (b) and seminal fluid (s) of various invertebrates

Spermatozoa (species 1 to 25) or erythrocytes (species 26-34) of:	Fluids of:															
	1 b	4 s	6 b	12 b	13 s	14 b	16 b	18 b	19 b	21 b	22 b	23 b				
POLYCHAETS																
1. <i>Chaetopterus variopedatus</i>	0	0	0	0	+	0	0	+	+	+	+	+	0	0	0	0
2. <i>Halosydna johnsoni</i> . . .	0	0	0	0	+	0	0	+	+	+	+	+	0	0	0	0
3. <i>Sabellaria californica</i>	+	+	0	0	0	0	0	0	0	+	+	+	0	0	0	0
ECHIUROIDS																
4. <i>Urechis caupo</i>	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+
5. <i>Thalassema</i> sp.....	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+
AMPHINEURANS																
6. <i>Mopalia muscosa</i>	+	+	0	0	0	+	0	+	+	0	0	0	0	+	+	+
7. <i>Ischnochiton magdalensis</i>	+	+	0	0	0	+	+	+	0	0	0	0	+	+	+	+
GASTROPODS																
8. <i>Acmea digitalis</i>	+	+	+	0	0	0	0	0	0	+	0	0	0	+	+	+
9. <i>Lottia gigantea</i> . . .	+	+	+	0	0	0	0	+	0	+	+	0	0	+	+	+
10. <i>Tegula galena</i>	+	+	+	0	0	0	0	0	0	+	+	+	+	+	+	+
11. <i>Astraea undosa</i>	+	+	+	0	0	+	0	0	0	+	+	+	+	+	+	+
12. <i>Megathura crenulata</i> . .	+	+	+	0	+	0	0	+	+	0	0	+	+	+	+	+
PELECYPOD																
13. <i>Mytilus californianus</i> . .	+	+	0	0	+	0	0	0	0	+	+	+	+	+	+	+
ECHINOIDS																
14. <i>Strongylocentrotus purpuratus</i>	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0
15. <i>S. franciscanus</i>	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0
16. <i>Lytechinus pictus</i>	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0
17. <i>Dendraster excentricus</i> ...	0	0	0	0	+	0	0	+	0	0	0	0	0	0	0	0

TABLE I—Continued

Spermatzoa (species 1 to 25) or erythrocytes (species 26-34) of:	Fluids of:											
	1 b s	4 b s	6 b	12 b s	13 b s	14 b s	16 b s	18 b s	19 b s	21 b s	22 b s	23 b s
ASTEROIDS												
18. <i>Patiria miniata</i>	+	+	0	0	0	+	+	0	0	0	0	0
19. <i>Pisaster ochraceus</i>	+	+	0	0	0	+	+	0	0	0	0	0
20. <i>Astropecten armatus</i>	+		0		0			0	0	0	0	0
HOLOTHURIID												
21. <i>Stichopus californicus</i>		0		0	0	0	0	0	0	0	0	
ASCIDIANS												
22. <i>Ciona intestinalis</i>	+	+	0	0	+	0	0	0	0	+	0	0
23. <i>Styela barnharti</i>	+	+	0	0	+	0	0	0	0	+	0	0
24. <i>Ascidia ceratodes</i>	+	+	0	0	0	0	0	+	+	+	0	0
FISH												
25. <i>Leuresthes tenuis</i>	0	0		0	0	0	0	0	0	+	+	+
26. <i>Girella nigricans</i>	0				0	0	0	0	0	+	+	
AMPHIBIA												
27. <i>Rana catesbeiana</i>	+	+										
28. <i>Bufo halophilus</i>	+		0	0	0	0		0	0	0	+	+
REPTILE												
29. <i>Sceloporus occidentalis</i>	+	0				0	0	0	0	+	+	0
BIRD												
30. Chicken	+	0		0	+	0		+	0	+	0	0
MAMMALS												
31. Guinea pig					+	+	0	+				
32. Rabbit					+		0					
33. Sheep	+	0		0	+	+	0	0	+		0	0
34. Man					+		0	+				

none of the twelve species examined were found to possess agglutinating action on all of the species that were tested.

With one exception no heteroagglutination reactions occurred with body-fluids and cells of animals belonging to the same taxonomic class. The exception to this consists in the agglutination of *Sabellaria* sperm by *Chaetopterus* fluid. It is of interest in this connection that these two genera are placed (Pearse, 1936) in separate subclasses (cryptocephala and phanerocephala respectively) of the polychaets. *Sabellaria* also differs from the other two polychaets that were tested in that its cells fail to agglutinate in the fluid of the mussel (13), sea-urchins (14, 16) and sea cucumber (21) as well as that of the lobster previously (Tyler and Metz, 1945) reported.

Another feature of the results is that closely related species behave alike with respect to the ability or inability of their cells to be agglutinated by the various body-fluids. This was evident in the previously reported experiments with lobster serum.

In the present data differences are observed between species belonging to different orders, as in the case of the two species (8 and 9) belonging to the docoglossid gastropods which differ from the three rhipidoglossid species (10, 11, and 12) in their reactions to two of the fluids (6 and 21). With the other twenty-nine species tested, similarity in behavior is exhibited by members of the same class or sub-class.

The tests with the diluted seminal fluids gave results that in most cases paralleled those obtained with the body-fluids. Eight exceptions (1 on 29; 4 on 9 and 12; 12 on 6; 13 on 11; 18 on 8 and 30; 21 on 14) may be noted in Table I out of a total of 186 cross-combinations in which both seminal fluid and body-fluid were examined. These exceptions are all in the same direction; namely, a failure of the dilute seminal fluid to cause agglutination while the corresponding body-fluid is active. It can, then, be stated that in those cases in which the diluted seminal fluid is found to possess heteroagglutinating activity, the corresponding body fluid is likewise found to be active. Thus, similarly acting heteroagglutinins are found in both body-fluid and seminal fluid. The above-mentioned few exceptions may be attributed to failure to obtain, in certain seminal fluid preparations, sufficient concentration of a particular heteroagglutinin to produce a visible reaction with the cells of some species. However, the tests necessary to determine the validity of this explanation have not, as yet, been made.

From the similarity in action of seminal fluid and body-fluid, it might be inferred that the activity of the former is due to contamination with the latter. However, it may be noted that in most of the species employed for preparation of seminal fluid (e.g., 4, 12, 14, 16, 18, 19, 21, 22) the sperm is readily obtained without any appreciable admixture of body-fluid. Another interpretation is that seminal fluid is normally similar to body-fluid in composition. Against this may be cited the fact that readily recognizable constituents of body-fluid, such as hemocyanin in the mollusks, are not observed in the seminal fluids. A third possibility is that identical heteroagglutinins are present in both fluids. However, serological similarity does not imply entirely identical molecular constitution. Reaction with a specific antigen implies similarity only on the part of the specific combining groups of the antibodies from diverse sources. In the present case it has not been shown that the heteroagglutinin in seminal fluid and that in body-fluid both react with the same antigenic group or structure on the sperm that they agglutinate. However, the generally parallel behavior of the two fluids, when tested with spermatozoa of different species, favors that view.

Absorption tests, to determine whether or not more than one heteroagglutinin is involved in the action of a particular fluid, were carried out with *Patiria* body-fluid. These were done in the manner previously described (Tyler and Metz, 1945). Before being used for absorption the sperm were washed repeatedly in order to free them of agglutinins contributed by the seminal fluid, and this was checked in each test by examination of the supernatant of an aliquot part of the sperm for agglutinating activity.

Samples of *Patiria* body-fluid were absorbed with sperm of six species of animals and tested for agglutinating activity on sperm of nine species. The results are given in Table II. This limited set of tests reveals the presence of at least four distinct heteroagglutinins in *Patiria* body-fluid. These evidently comprise:—one for the two polychaets, one for the two echiuroids and *Mytilus*, one for the two gastropods, and one for the two ascidians. It seems likely, then, that the situation in *Patiria* body-

fluid is similar to that previously reported for the lobster; namely, the presence of a number of distinct agglutinins, each with broad group specificity.

DISCUSSION

Heteroagglutinins are evidently normal constituents of the body-fluids of animals. They have generally been considered to be non-specific agents. However, from the fact that the fluids of various species act on the cells of different assemblages of other species, the heteroagglutinins must be regarded as having some degree of specificity. The previously reported (Tyler and Metz, 1945) absorption tests with lobster-serum gave evidence of the presence of ten distinct heteroagglutinins which are, for the most part, each specific for a taxonomic class of animals. The present results with *Patiria* body-fluids are indicative of similarly broad specificity on the part of the four heteroagglutinins found therein. It is clear, however, that heteroagglutinins of different species and also different heteroagglutinins of the same animal may differ considerably in the range of species on which they act. The rule is

TABLE II

Agglutinative activity of Patiria body-fluid after absorption with spermatozoa of various species

Spermatozoa of:	Body-fluid absorbed with washed sperm of:					
	Chaetopterus	Urechis	Thalassema	Astraea	Megathura	Mytilus
Chaetopterus ..	0	+	+	+	+	+
Halosydna.....	0	+	+	+	+	+
Urechis.....	+	0	0	+	+	0
Thalassema...	+	0	0	+	+	0
Astraea.....	+	+	+	0	0	+
Megathura.....	+	+	+	0	0	+
Mytilus.....	+	0	0	+	+	0
Ciona.....	+	0	0	+	+	+
Ascidia..	+	0	0	+	+	+

that closely related species react alike to a particular fluid, but the closeness of relationship required depends upon the particular heteroagglutinating fluid employed. In the present tests (Table I) we find that species that belong to the same class, in most instances, behave alike with respect to the ability or inability of their cells to be agglutinated by the body-fluids of all twelve of the species examined.

In the sera of various mammals natural heteroagglutinins are found (cf. Thomsen, 1932; Wiener, 1943; Landsteiner, 1945) that are relatively species-specific and in human sera, as is well known, natural isoagglutinins are encountered that differentiate groups of individuals. In the various invertebrate body-fluids examined, agglutinins of such specificity have not, as yet, been found, the fluid of a particular species being inactive on cells of closely related species. However, from the ripe gametes, of many of these species natural agglutinins are obtained that act within the species. These consist in the fertilizins from eggs and antifertilizins from sperm described by Lillie (1913 et seq.), Just (1930), Frank (1939), Tyler (1939 et seq.), Hartman *et al.* (1939 et seq.), Runnström *et al.* (1942 et seq.) and others. While these agents act on gametes of the opposite sex within the species, they also have

been found to act on closely related species and in some instances the preparations act on remotely related species. Thus *Arbacia* egg water was found (Lillie, 1913) to agglutinate *Nereis* sperm, and sea-urchin eggs have been found (Runnström *et al.*, 1942) to be agglutinated by sperm extracts of animals as remotely related as the salmon and the ox. In Lillie's experiment it was shown that absorption with *Nereis* sperm removed the cross-reacting substance from *Arbacia* egg water without diminishing its agglutinating action on *Arbacia* sperm. Similar absorption experiments have not been reported in most of the cross-heteroagglutination reactions obtained by later workers with fertilizin and antifertilizin preparations. The need for such experiments is quite evident before any adequate determinations can be made of the specificity of these interacting substances obtained from the gametes. The present results may provide a helpful basis of procedure in such experiments.

The bearing of the heteroagglutination reactions on phylogenetic questions has been previously (Tyler and Metz, 1945) discussed. The present results are consistent with the previously expressed view that the reactivity of the cells of a particular species with various fluids is a characteristic of a group of related species and constitutes a group-specific trait in addition to the various group-specific morphological and chemical features of animals. There is no reason, as yet, for considering this trait to be of more general significance than any other in any applications that might be made to phylogenetic problems.

SUMMARY

1. The body-fluids of 12 species of invertebrates (including two ascidians) and the seminal fluids of 11 species were examined for agglutinating action on the spermatozoa or blood cells of 34 species of animals.
2. All of the fluids were found to contain agglutinins for the cells of some of the species tested. Five of the fluids gave reactions with most of the species but none reacted with all of the species.
3. With one exception no heteroagglutination reactions were obtained with fluids and cells of animals belonging to the same taxonomic class.
4. Closely related (same class in most cases or same order in some) species were found to behave alike with respect to the ability or inability of their cells to react to the various fluids, and the fluids of closely related species exhibited similar reactivity.
5. The diluted seminal fluids gave reactions that in most cases paralleled those obtained with the body-fluids.
6. Absorption tests revealed the presence of at least four distinct heteroagglutinins in *Patiria* body-fluid, and indicated that each is characterized by a broad group-specificity similar to that previously reported for lobster-serum.
7. The general bearing of these results on fertilizin-antifertilizin reactions and on phylogenetic problems is briefly discussed.

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DISTRIBUTION AND PROPERTIES OF INTRACELLULAR ALKALINE PHOSPHATASES¹

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The chemical and structural composition and the interrelationships of the components of the cell have long been the concern of biologists, in hope of revealing the fundamental processes within the structural and functional unit of all living matter. The early studies of Miescher upon the chemical composition of the nucleus were contemporary with the studies of the structure of the nucleus by van Beneden, Flemming, and others, which later culminated in the firm establishment of the nucleus as the site of phenomena of central importance in the mechanism of Mendelian heredity. The biochemical line of investigation, started by Miescher, has had no such tradition of continuous progress as have the studies of chromosomal structure; but as a result of sporadic advances following the appearance of new methods and techniques, certain facts about the chemical composition of the cell and the nucleus have become firmly established. Miescher noted that the nucleus had a high content of organically bound phosphate; later researches (Levene and Bass, 1931) have fully confirmed this, and have led to a fairly comprehensive knowledge of the chemical composition of nucleic acid, the substance in which all this nuclear phosphate is contained. The unit of nucleic acid is the mononucleotide, a phosphoric acid ester of pentose sugar in glucosidic linkage with one of the purine or pyrimidine heterocyclic bases. The nucleic acids, which have been intensively studied, have been found to be polynucleotides with four different bases, and in their native form they are very highly polymerized, consisting in some cases of hundreds or thousands of nucleotides. There are two types of nucleic acid with respect to the sugar; one contains a pentose which whenever identified has been found to be d-ribose, and the other a desoxypentose found to be d-ribodeseose. The wide application of the Feulgen cytochemical test for desoxypentose indicates that nucleic acid with this sugar does not occur outside of the chromatin of the cell nucleus (that is, in the chromosomes), and actual analysis of isolated nuclei and chromosomes has confirmed the view that desoxypentose is the characteristic nucleic acid of chromatin (Mirsky and Pollister, 1942). The name chromonucleic acid has recently been proposed (Pollister and Mirsky, 1943) for desoxypentose nucleic acid as a convenient way of emphasizing this striking limitation in distribution. Nucleic acids can also be located within the cell by means of the intense specific absorption of ultra-violet light by purine and pyrimidine bases, and Caspersson (1936, 1940) has made use of a combination of microspectroscopy and the Feulgen nucleal reaction to determine the distribution of the Feulgen negative pentose nucleic acids. This was found to be largely in the cell cytoplasm, the occurrence of pentose nucleic acid in the nucleus being confined to the plasmosome,

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or the non-chromatin nucleolus. The term plasmonucleic acid has been proposed to embody this information concerning the distribution of pentose nucleic acid. The nucleic acid makes up but a part, usually less than half, of the content of the nucleus. The remainder is apparently largely protein (Mirsky and Pollister, 1942). Much of this is of the basic type, either histone or protamine, but there is also evidence of protein of globulin type (Caspersson, 1936; Mazia and Jaeger, 1939).

In view of the fact that the nuclear chromatin contains a large amount of phosphate in desoxyribose nucleic acid, it is of considerable interest that definite evidence of phosphatase activity within chromatin has been found. Almost simultaneously the author (Krugelis, 1942) and Willmer (1942) reported the demonstration of alkaline phosphatase activity within chromosomes. This discovery stimulated the present study, the purpose of which is two-fold. The first purpose was to investigate the occurrence of alkaline phosphatase in all parts of the cell, and to investigate further the correlation of desoxyribose nucleic acid with alkaline phosphatase activity in the chromosomes. The material which seemed most promising for this first part of the study were the salivary gland chromosomes of *Drosophila* larvae, because of their peculiar structure which manifests itself in bands or regions rich in desoxyribose nucleic acid, alternating with interband regions poor or lacking in desoxyribose nucleic acid. This part of the investigation can be expressed in the form of a question: Is phosphatase activity located in the chromosomal regions rich in desoxyribose nucleic acid? The second part of the investigation followed from the first, and included the use as substrate of actual chemical substances that are found in the cell, in order to discover whether alkaline phosphatase is capable of hydrolyzing naturally-occurring substances with which its activity seems to be physically associated. If the activity of phosphatase is located in regions rich in desoxyribose nucleic acid, is it capable of hydrolyzing desoxyribose nucleic acid and other naturally occurring substances in the cell? This part of the investigation it was hoped would lead to some suggestion as to the possible role of phosphatase activity within the cell and the chromosome.

METHODS AND MATERIALS

The histochemical method used was that developed by Gomori (1939, 1941); when using smeared insect material it was found necessary to make some modifications of the preliminary steps. This process involved dissecting, and smearing the insect organs in isotonic saline, or Ringer's, made according to Buck and Melland (1942). After smearing, the material was fixed in 95 per cent ethyl alcohol vapors for one hour, then fixed in liquid 95 per cent ethyl alcohol for two hours. Material fixed as short a time as one hour and as long a time as 48 hours showed little appreciable difference in the results. The coverslips fell off readily in the alcohol, and the smears on the slides were washed briefly in distilled water and then incubated in the substrate solution. The mammalian organs used, namely, the kidney, liver, testis, intestine, and pancreas, were removed after ether anesthesia, fixed in 95 per cent ethyl alcohol for 24 hours, dehydrated in 100 per cent ethyl alcohol, cleared in benzene, and embedded in paraffin in the usual manner. Sections of 5 microns thickness were cut. To avoid any variation in mounting or incubating, sections of the five organs were mounted together on one slide. After deparaffinization, hydration, and washing, sections were ready for immersion and incubation in the substrate solution.

The process from this point on was the same for both the sectioned and the smeared materials. The substrate solution was of the following composition :

- 2 parts of 0.1 M sodium glycerophosphate (or 0.1 M other organic phosphate ester)
- 2 parts of 0.1 M calcium nitrate
- 1 part of 0.1 M veronal buffer at pH 9.4
- 5 parts of distilled water.

The pH was checked with thymol blue and adjusted to pH 9.0 to 9.2 with NaOH. The incubation was carried out at 25–28° C. and lasted from 12 to 24 hours. Following the incubation in the substrate, the slides containing the smears or the sections were immersed in 0.02 M calcium nitrate for one minute, then into 0.1 M cobaltous nitrate for two minutes, then dilute potassium sulfide for two minutes. After this visualization treatment, slides were washed, dehydrated, cleared, and mounted by the usual histological technique. The results were checked, in some cases, by parallel experiments in which the silver nitrate visualization method (Gomori, 1939) was used. When no activity was obtained in the substrate-incubated slides, the reaction was allowed to proceed for 72 hours to verify the negative results.

At the suggestion of Dr. A. E. Mirsky partially depolymerized nucleic acid was tried as the substrate. This depolymerization was accomplished by using desoxyribonuclease from beef pancreas, made according to the method of McCarty (1946). Desoxyribose nucleic acid is dissolved in distilled water and then placed in a nuclease medium of the following composition :

- 0.01 per cent magnesium sulfate
- 0.003 M gelatin
- 0.025 M veronal-HCl buffer at pH 7.5.

The nuclease is allowed to react upon the desoxyribose nucleic acid for 24 hours in which time the solution becomes clear and loses its viscosity. This change is used as a criterion for the depolymerization of the nucleic acid. This depolymerization leaves the nucleic acid in only a less highly polymerized state; and does not, for example, reduce it to an approximation of tetranucleotide. After the depolymerizing reaction, the solution is heated to 60° C. for fifteen minutes to destroy the activity of the nuclease on the desoxyribose nucleic acid, and then is used as the substrate source of phosphate ester.

The biological materials used in this study were insect and mammalian organs. The salivary glands of the larvae of *Drosophila* were used for the first part of the experimentation. The larvae were raised at 17–18° C. and at room temperature. The glands were dissected out when the larvae were full grown and had left the food prior to pupation. The species of *Drosophila* investigated included the Brazilian species *Drosophila pallidipennis* obtained through the generosity of Professor T. Dobzhansky, *D. simulans*, *D. virilis*, and *D. ananassae* obtained from the stocks at the Biological Laboratories, Cold Spring Harbor, N. Y. The major part of the investigation on *Drosophila* was done on *Drosophila melanogaster*.

The mammalian organs used were those of mice obtained from the stocks maintained at Columbia University by Professor L. C. Dunn and Dr. S. Gluecksohn-Schoenheimer. The testes were used either after being smeared in the body fluid surrounding the tubules, or after being fixed and embedded as the other organs were.

Yeast nucleic acid and adenylic acid were obtained from the Schwartz Laboratories, New York; thymonucleic (desoxyribose nucleic) acid and nuclease were generously supplied by Dr. A. E. Mirsky of the Rockefeller Institute for Medical Research; guanylic acid, sodium guanylate, and cytidylic acid were obtained from the Levene collection of chemicals at the Rockefeller Institute, also through the kindness of Dr. A. E. Mirsky. Photographic materials were provided from a grant to Columbia University by The Rockefeller Foundation.

EXPERIMENTAL DATA

Intracellular occurrence and localization of alkaline phosphatase activity in the salivary gland cells of Drosophila melanogaster

Upon microscopic examination of smears of larval salivary glands of *Drosophila*, it is evident that in the preparations which have been incubated in the substrate, there is a much greater precipitate of cobaltous sulfide, indicating alkaline phosphatase activity, than in the smears incubated in control solutions. A diffuse precipitate occurs throughout the cytoplasm, but there is a much higher density within the nucleus (Fig. 1). This agrees with the observations of Moog (1944), on the tissues of the chick embryo that the nuclei were never less reactive than the cytoplasm and that the nuclei, unlike the cytoplasm, were never negative in reaction. Dounce (1943) using nuclei isolated from rat liver, also found that alkaline phosphatase activity is much greater in nuclei than in total liver.

Within the nucleus the precipitate is sharply localized in the chromosomes and nucleolus. The nuclei of the control slides show slight, if any, precipitate, with the exception that there is always some cobaltous sulfide in the control nucleoli (Fig. 2), but this is always considerably less in amount than that in nucleoli of substrate-incubated preparations.

The most dense precipitate is found within the chromosomes. When smeared in Ringer's solution, which is necessary to preserve the activity of the enzyme, the details of the chromosome structure are by no means as distinct as in the acetic acid smears that have been used in mapping bands. Nevertheless one can be certain that the cobaltous sulfide within the chromosome is concentrated in transverse bands, alternating with regions of very little or no precipitate (Figs. 3 and 5). This observation was reported earlier (Krugelis, 1945) and it has recently been confirmed by Danielli and Catcheside (1945) who compare the individual bands with specific regions of the cytogenetic chromosomal maps. In the preparations of Figures 3 and 5 the identity of the regions of phosphatase precipitate with the bands of acetic acid smears was established by a direct cytological observation. This method made use of the fact that the cobaltous sulfide precipitate is not altered by the standard Feulgen cytochemical procedure, that it becomes possible to superimpose the nucleal reaction on a phosphatase test. Figure 4 is from such a preparation; and comparison with Figure 5, the same chromosome after the phosphatase reaction but before the Feulgen test, shows that the bands in which the enzyme activity is localized correspond strictly with the Feulgen positive bands that form the basis of the familiar cytogenetic maps. The superposition of the second procedure merely changes the brown band to a purple brown, which is clearly a combination of cobaltous sulfide and basic fuchsin. This combination of the two cytochemical tests shows, in a vivid manner, that the highest level of alkaline phosphatase activity within the salivary gland cell occurs

in exactly the region that contains a very high concentration of organic phosphate in the form of desoxyribose nucleic acid.

Properties and distribution of intracellular alkaline phosphatases

The occurrence of the enzyme activity and the desoxyribose nucleic acid within the same parts of the salivary gland chromosome suggests a functional relationship, which suggestion was put to test by using other phosphate esters as substrates for the enzyme activity. In order to elucidate a more specific picture of possible enzyme activity *in vivo* within the nucleus and the cell, esters were chosen which are normally present in the cell, and which the enzyme would consequently be expected to hydrolyze. The cellular substrates used, along with sodium glycerophosphate as the standard, were purine and pyrimidine nucleotides, ribose nucleic acid (presumably not highly polymerized), and desoxyribose nucleic acid (both polymerized and partially depolymerized). These substrates were tried on various mouse tissues for the presence of the enzyme activity in the cells. The testis was used for the chromosomal localization, since the other tissues contained nuclei which were mainly in the resting stages. In the results the black-brown precipitate of cobaltous sulfide indicates the site of alkaline phosphatase activity on the specific organic substrate in the incubating solution, and the density of the precipitate may be regarded as a measure of the intensity of the enzyme activity.

The photomicrographs (cf. Plates II, III, and IV) illustrate the results of these experiments; and roughly quantitative estimates of the intensities of the reaction are summarized in Table I. With sodium glycerophosphate and the nucleotides as substrates, the precipitate is not sharply localized, though it is always more dense in the nucleus and chromosomes than in the cytoplasm. By contrast, the reactions with depolymerized nucleic acids result in precipitates which are restricted to particular parts of the cell. On the slide in which depolymerized chromonucleic (desoxyribose nucleic) acid has been used as substrate the dark nuclei and chromosomes stand out very distinctly against a colorless cytoplasmic background, an appearance not unlike that of a Feulgen preparation. On the other hand, if plasmonucleic (ribose nucleic) acid is the substrate, the slide as a whole is intensely dark due to cytoplasmic precipitate, and the nuclei appear as light areas. The two types of slides compare with one another somewhat as do a photographic positive and negative. The chromonucleic acid precipitate is not uniformly distributed throughout the

EXPLANATION OF PLATE I

The dark precipitate indicates the phosphatase activity. Photomicrographs made from smear preparations. Figures 1, 3-5 show phosphatase activity with sodium glycerophosphate as the substrate.

FIGURE 1. Alkaline phosphatase activity in salivary gland of *Drosophila melanogaster* larva. Magnification is 200 X.

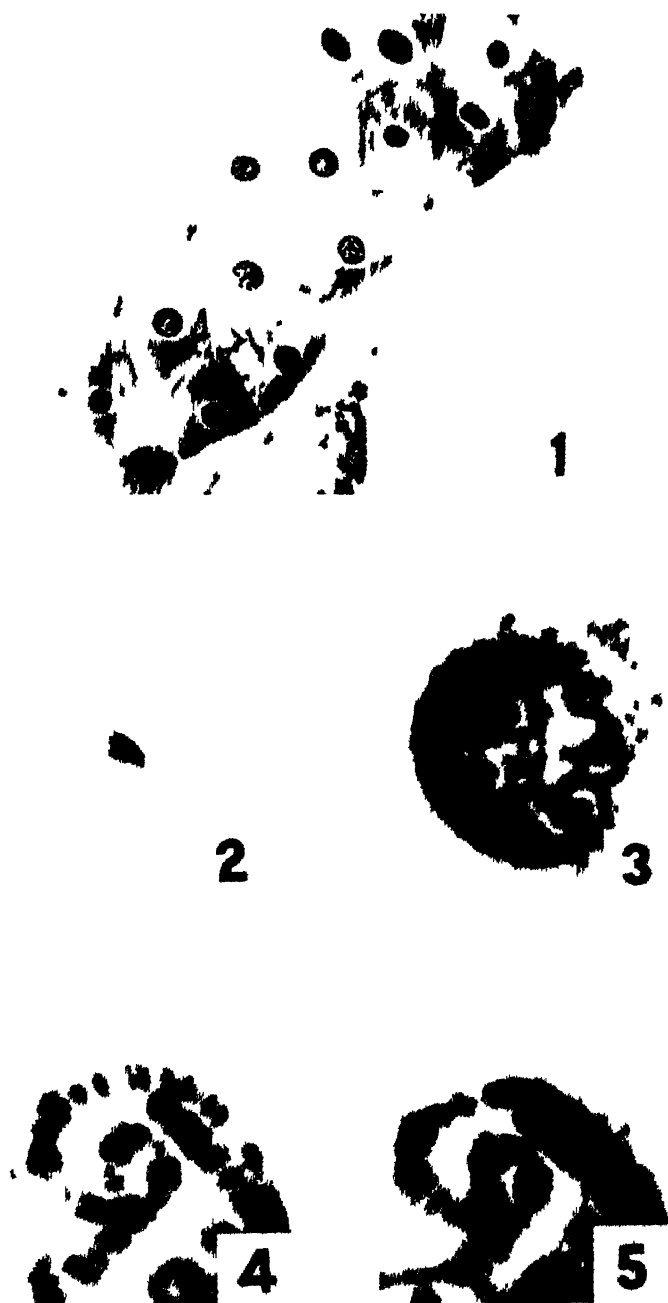
FIGURE 2. Salivary gland nucleus from control slide which had no substrate for the alkaline phosphatase activity. Magnification is 1000 X.

FIGURE 3. Salivary gland nucleus showing alkaline phosphatase activity. Magnification is 1000 X.

FIGURE 4. Salivary gland chromosomes with the Feulgen reaction applied over the phosphatase reaction precipitate. Magnification is 3000 X.

FIGURE 5. Salivary gland chromosomes (same chromosomes as Fig. 4) showing alkaline phosphatase activity before Feulgen reaction applied over the precipitate. Magnification is 3000 X.

PLATE I



nucleus. Instead it occurs only within the formed structures of the nucleus, the chromatin and the plasmosome nucleolus.

In an attempt to find enzyme differences involved in producing such specific localizations of alkaline phosphatase activity, these reactions were subjected to various conditions which might alter the reaction environment. Exposures to temperatures of 55° C. or over for a period of 5 minutes or longer produced complete irreversible inactivation of all the enzyme reactions. Magnesium ions in the concentration of 0.01 M magnesium sulfate showed some activation with the cytoplasmic reaction on ribose nucleic acid, and little or no increase on the nuclear and the general reactions. This very slight activating effect is in agreement with Schmidt and Thannhauser's (1943) observation that there is but slight effect of magnesium on intestinal alkaline phosphatase activity with sodium glycerophosphate as substrate.

TABLE I
Phosphatase reaction with different substrates

Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate	1	2	1	2	0	2	±1	2	3	2
Adenylic acid, guanylic acid, cytidylic acid	1	2	1	2	0	2	0	2	1	2
Desoxyribose nucleic acid	0	0	0	0	0	0	0	0	0	0
Depolymerized desoxyribose nucleic acid	0	2	0	2	0	2	0	2	1	2
Ribose nucleic acid	2	±1	2	1	1	±1	2	±1	2	1

Density of cobaltous sulfide precipitate recorded from visual estimates. 0, no reaction; ± doubtful; 1, definite reaction; 2, strong reaction; 3, very strong reaction. Each record based on microscopic examination of at least 20 slides. Column c, cytoplasmic precipitate; column n, nuclear precipitate.

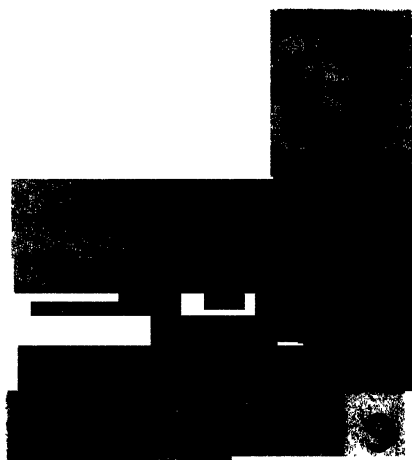
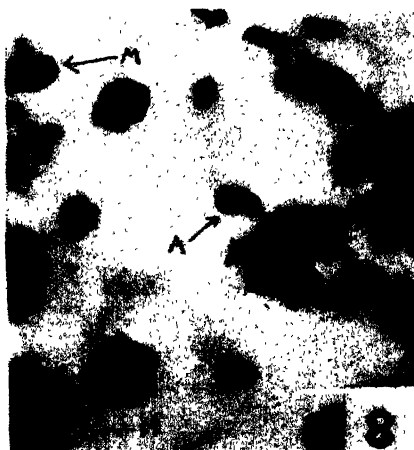
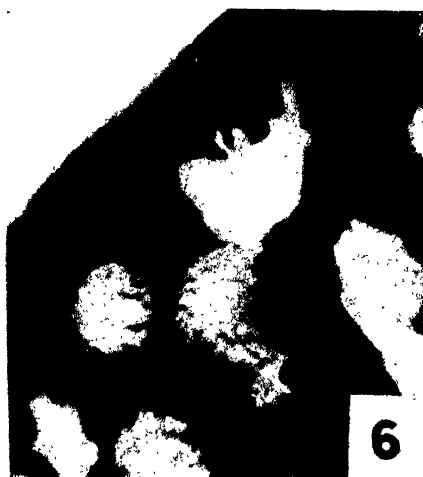
Cyanide ions in the concentration of 0.01 M potassium cyanide completely inhibited all three types of reaction.

Arsenate ions in the concentration of 0.01 M sodium arsenate showed a more selective effect on the different reactions than did the environmental influences mentioned above. In the presence of arsenate, the alkaline phosphatase activity with the nucleic acids as substrates is completely suppressed. The glycerophosphate splitting, by contrast, in most cases proceeds at its normal rate in a medium which contains arsenate. A roughly quantitative estimation of the inhibition or suppression of activity by arsenate ions is presented in Table II.

DISCUSSION

The differences in site of alkaline phosphatase activity in the same tissues when using different phosphate bearing substrates indicate localized reactions of three

PLATE II



Photomicrographs from 5 micra thick sections. Figures 6-8 show alkaline phosphatase activity with sodium glycerophosphate as substrate.

FIGURE 6. Small intestine of mouse showing activity of the enzyme distributed in the mucosa and submucosa layers. Magnification is 1000 \times .

FIGURE 7. Testis of mouse. Magnification is 1000 \times .

FIGURE 8. Testis of mouse. *M* indicates chromosomes in metaphase. *A* indicates chromosomes in anaphase. Magnification is 1000 \times .

FIGURE 9. Testis of mouse from a control slide with no substrate for enzyme activity applied. *M* indicates chromosomes in metaphase. Magnification is 1000 \times .

types, as follows: first, a general alkaline phosphatase reaction in both the cytoplasm and the nucleus, as in the case where sodium glycerophosphate and nucleotides are used as substrates; second, a definite nuclear reaction with little or no activity in the cytoplasm, as in the case of depolymerized desoxyribose nucleic acid as substrate; third, a definite cytoplasmic reaction with little reaction in the nucleus, as in the case of ribose nucleic acid as substrate. Since the enzymes cannot be isolated at this time, and since the nuclear and the cytoplasmic reactions are not absolutely specifically nuclear or cytoplasmic, they will be listed as reaction types with reference only to the location of the activity observed. With the different substrates used, the total reaction of phosphatase activity is somehow produced, and this is detected by the location of the cobaltous sulfide precipitate. The final reactions produced under a

TABLE II

Intensity of precipitate of phosphatase reaction in different tissues using different substrates with and without arsenate ions

Substrate	Intestine		Testes		Liver		Pancreas		Kidney	
	c	n	c	n	c	n	c	n	c	n
Sodium glycerophosphate										
No arsenate ions . . .	1	2	1	2	0	2	±1	2	3	2
With arsenate ions	1	1*S1	1	2	0	0*S3	±1	2*S0	2	±1*S1
Depolymerized desoxyribose n. acid										
No arsenate ions	0	2	0	2	0	2	0	2	1	2
With arsenate ions	0	0*S3	0	0*S3	0	0*S3	0	±1*S2	0	0*S3
Ribose nucleic acid										
No arsenate ions	2	±1	2	1	1	±1	2	±1	2	1
With arsenate ions	0	0*S3	2	0*S3	0	0*S3	0	0*S3	2	1*S0

*S = suppression estimates.

S0 is no suppression.

S1 is slight suppression.

S2 is much suppression.

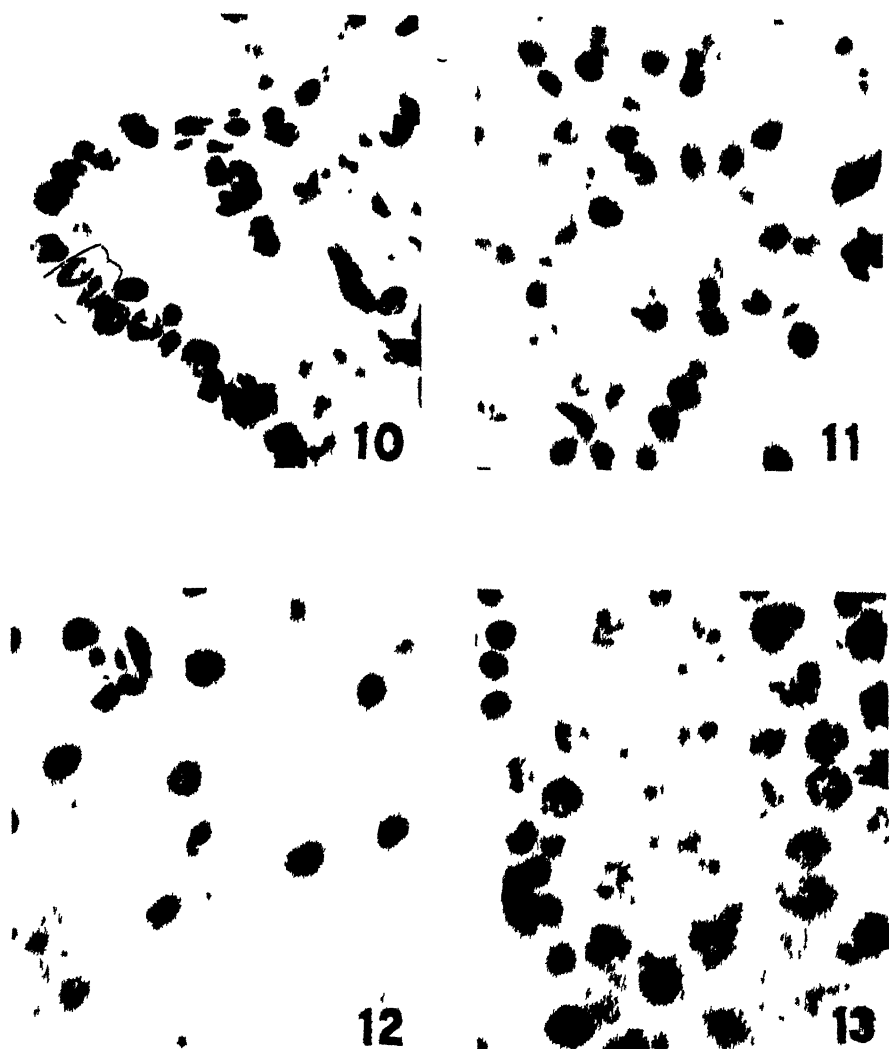
S3 is complete suppression.

Each record represents observation on 8 experimental slides.

variety of substrate conditions must be due to at least three different enzymes, working in at least two different complexes.

The suggestion that the reactions observed are due to complexes of enzymes is based on the chemical structure of nucleic acids. The ribose and desoxyribose nucleic acids, which were applied as substrates, are polymers of mononucleotides, which are considered to be linked to each other by an ester bond between the phosphate group of one nucleotide and the sugar group of the neighboring nucleotide, thus making the nucleic acids diesters of phosphoric acid all along the chain except in the terminal monoester of phosphoric acid. The total phosphatase action on the nucleic acids might be considered as essentially due to a specific desoxyribose nucleic acid phosphodiesterase, and a specific ribose nucleic acid phosphodiesterase action liberating mononucleotides of the nucleic acids, which then are hydrolyzed by a phospho-

PLATE III



Photomicrographs from 5 micra sections. Figures 10-13 show alkaline phosphatase activity with depolymerized deoxyribose nucleic acid as substrate.

FIGURE 10. Small intestine of mouse showing the distribution of the enzyme activity in the mucosa layer. Magnification is 1000 \times .

FIGURE 11. Kidney of mouse showing enzyme activity in the proximal tubules seen in cross-section. Magnification is 1000 \times .

FIGURE 12. Pancreas of mouse showing distribution of enzyme activity. Magnification is 1000 \times .

FIGURE 13. Testis of mouse showing enzyme activity. Magnification is 1000 \times .

monoesterase to liberate the inorganic phosphate. Thus the precipitate formed when the nucleic acids are applied as substrates is due to the activity of at least two different enzyme complexes, as follows: in the *cytoplasm*, first, a specific phosphodiesterase ("plasmonucleodiesterase") liberates ribonucleotides; second, phosphomonoesterase liberates inorganic phosphates from these nucleotides; in the *nucleus*, first, a specific phosphodiesterase³ ("chromonucleodiesterase") liberates desoxyribosenucleotides; second, phosphomonoesterase liberates inorganic phosphate from these nucleotides. This specificity of the diesterase follows from the fact that ribose nucleic acid will not serve as substrate for the nuclear diesterase activity, nor will desoxyribose nucleic acid serve for the cytoplasmic activity. With regard to the monoesterase activity, however, no such specificity has been detected, for the products of ribose nucleic acid hydrolysis serve equally well as substrates for phosphate production in either nucleus or cytoplasm.

While the action of the phosphodiesterase in freeing mononucleotides from lower polynucleotides (depolymerized nucleic acid) presumably can only occur by attack upon the linkage between the phosphate of one nucleotide and the sugar of the adjacent unit, it is evident that there is considerable restriction upon the exact nature of the bond which can be so attacked. If the enzyme were able to hydrolyze the bond at many points along a nucleic acid chain (consisting, let us say, of 2000 nucleotides) the diesterase should also function as a depolymerase, and a phosphate precipitate should be formed when the long, polynucleotide chains (polymerized nucleic acid) are used as substrate. Even after 72 hours action, however, there is no visible precipitate under these conditions, in contrast to the depolymerized nucleic acid experiments in which a dense precipitate is formed in a few hours. The type of bond which the diesterase can attack is evidently one which is enormously multiplied by a process of depolymerization. A most obvious view of enzyme specificity that would agree well with these facts is that the diesterase can attack only the bond between a terminal nucleotide and the penultimate nucleotide. Considering, for example, an extreme case, depolymerization of a 2000 unit polynucleotide chain to the minimum tetranucleotide should increase the number of bonds which such a specific diesterase can attack by a factor of 500. If specific terminal hydrolysis is the mechanism, the amount of mononucleotide that would become available by diesterase action upon the end of a highly polymerized nucleic acid chain would surely give an amount of phosphate precipitate so slight that it would be cytologically undetectable.

It is highly important to the question of the functional significance of the distribution of the phosphatases acting on the nucleic acids, that this actually coincides in a striking manner with the known locations of desoxyribose and ribose nucleic acids within the cell. The phosphatase activity on depolymerized desoxyribose nucleic acid is restricted to the chromatin of the nucleus, the only part of the cell in which this type of nucleic acid is found. By contrast, the ribose nucleic acid phosphatase activity is in the cytoplasm, a region in which only ribose nucleic acid has ever been demonstrated. (The only marked discrepancy is the occurrence of desoxyribose

³ Concerning the existence of this nuclear diesterase, there is some possible supporting evidence from the work by Mazia and Ballentine, reported by Mazia (1941), on an intranuclear enzyme from *Arbacia* eggs. Their enzyme, termed polynucleotidase, was active at a pH 9.0 and was capable of reacting on desoxyribose nucleic acid still in a polymerized form.

PLATE IV



Photomicrographs from 5 micra thick sections. Figures 14-17 show alkaline phosphatase activity with ribose nucleic acid as the substrate.

FIGURE 14. Small intestine of mouse showing the enzyme activity in the cells of the mucosa layer. Magnification is 1000 \times .

FIGURE 15. Small intestine of mouse showing goblet cells in the mucosa layer and the distribution of the enzyme activity. Magnification is 1000 \times .

FIGURE 16. Testis of mouse showing the enzyme activity. A denser precipitate is found in the nuclei of testis cells than in nuclei of other tissues under the conditions of the same substrate. Magnification is 1000 \times .

FIGURE 17. Kidney of mouse, showing enzyme activity in the proximal tubules in cross-section. Magnification is 1000 \times .

nucleic acid phosphatase activity in the nucleolus, which, since it is Feulgen negative, is considered to contain ribose nucleic acid.)⁴

Certain possible *in vivo* functions of these alkaline phosphatases are at once obvious. Not only can the nuclear diesterase split off terminal mononucleotides, as in these experiments, but in the reverse direction, it may conceivably catalyze the terminal growth in the development of nucleotide chains. One may easily picture the later stages of synthesis of a full length nucleic acid chain as involving the cooperation of two enzymes: the diesterase slowly builds up short chains by terminal growth and this is followed by the action of the depolymerase type of enzyme catalyzing the union of these short chains into the long complex which is such an important structural component of a chromosome.

While it is also obvious that catalysis of the synthesis of a mononucleotide from a nucleoside by phosphomonomesterase is an essential step in nucleic acid synthesis, one's attention here tends rather to focus on the possibilities of dephosphorylation of nucleotide as a source of energy for nuclear and cytoplasmic reactions. Thus energy for synthesis of chromosomes and their products may or may not be available according to whether the nucleotides are structurally isolated from phosphomonomesterase activity by being bound in nucleic acid chains, or whether as a result of a successive action of nuclear depolymerase, and "chromonucleodiesterase," there is mononucleotide available for dephosphorylation. Similarly the actual availability of energy for such cyclic nuclear mechanical processes as chromosome coiling and mitotic movement may be dependent upon a cycle of binding and release of mononucleotide from its nucleic acid storehouse.

SUMMARY

1. Using the histochemical test for alkaline phosphatase reaction in the larval salivary glands of several species of *Drosophila*, activity was found to be present in three main parts of the cell; the cytoplasm, the nucleolus, and the chromosomes.

2. Phosphatase activity was found rather generally distributed in both the cytoplasm and the nucleus. Within the larval salivary gland chromosomes, the enzyme activity was localized in those chromosomal regions which are Feulgen positive, and thus corresponds to the regions containing large concentrations of desoxyribose nucleic acid.

3. Different naturally occurring phosphate bearing substances were used as phosphatase substrates on mouse tissues, and resulted in demonstration of three different types of phosphatase reactions based on the localization of the enzyme activity.

- a) A general reaction with phosphatase activity located in both nucleus (nucleolus and chromosomes) and the cytoplasm was present when sodium glycerophosphate and nucleotides were used as substrates.

- b) No phosphatase reaction occurred on polymerized desoxyribose nucleic acid, but a specific nuclear reaction (nucleolus and chromosomes) was present when nuclease-depolymerized desoxyribose nucleic acid was used as a substrate.

⁴ We do not actually know the location of the possible substrates for the diesterase activity, since the methods for localizing nucleic acids do not, in all likelihood, preserve any but the high polymers, and it may well be that the lower polymers of the sort used as substrates are not the same in distribution as the larger complexes. In such a difference may lie the explanation of the discrepancies above.

c) A strong cytoplasmic reaction with slight reaction in the nucleus was present when ribose nucleic acid was used as a substrate.

4. Subjection to several environmental variables produced little further evidence as to the differences among these three types of localized reactions.

5. The three types of alkaline phosphatase reactions observed were suggested to be due to at least two phosphodiesterases and a phosphomonoesterase.

6. The nuclear phosphatase complex and the cytoplasmic phosphatase complex each probably consist of a specific phosphodiesterase, which splits the ester linkage between the phosphate of one nucleotide and the sugar of the neighboring nucleotide, and a phosphomonoesterase which splits the second ester linkage and liberates inorganic phosphate.

ACKNOWLEDGMENT

I wish to express my appreciation and gratitude to Professor H. Burr Steinbach for originally bringing to my attention the cytochemical possibilities of the alkaline phosphatase test; to Professor Arthur W. Pollister for his advice and encouragement throughout the course of the work and for his help in the preparation of the manuscript; to Dr. Alfred E. Mirsky for advice and essential materials; and to Dr. Robert Ballentine and Dr. Jack Schultz for many helpful suggestions.

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PHYSIOLOGY OF INSECT DIAPAUSE: THE ROLE OF THE BRAIN IN THE PRODUCTION AND TERMINATION OF PUPAL DORMANCY IN THE GIANT SILKWORM, PLATYSAMIA CECROPIA

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The phenomenon of insect diapause presents an exceptionally clear statement of one of the most important problems in biology; to wit, the nature of the factors that preside over cellular growth and differentiation. For with the onset of diapause and through the workings of internal physiological mechanisms still to be elucidated, growth suddenly comes to a standstill and the animal for months thereafter persists in a genuine state of suspended development. With the termination of diapause the rapid tempo of cellular activity returns and metamorphosis continues where it had left off. Thus whatever may be the inner mechanism for the induction and termination of diapause, it must have the capacity to turn morphogenesis off and on in a most striking way.

The study of this phenomenon has not failed to claim the attention of a large array of investigators. For example, even in 1932, Cousin was able to cite 347 papers in a review of the literature. That this extensive literature has so imperfectly advanced our knowledge of diapause is apparently due to the fact that most investigations have been carried out either on muscoid flies, which have a most imperfect and complex diapause, or on the eggs of silkworms and grasshoppers, which are too small to permit extensive manipulations of the individual animal. In the present investigation these difficulties were minimized by working on species of insects that possess a wholly characteristic pupal diapause, and, by virtue of weighing up to 8 grams per individual, are among the very largest insects in America.

MATERIALS AND METHODS

Pupae of the giant silkworm, *Platysamia cecropia*, were used for the most part, approximately 1200 pupae being studied in a total of 690 experiments. These insects were reared from eggs obtained from fertile females; a lesser number of pupae were secured from dealers. In my experience, this species has never failed to enter into diapause immediately after pupation, thus giving only one brood a year. If the pupae are maintained constantly at room temperature, diapause persists for not less than five months; if they are placed immediately after pupation at a temperature of 3° to 5° C. and chilled for 1½ months or longer, adult moths emerge about 1 to 1½ months after being returned to room temperature. For this reason the stock of material was divided at the outset into two batches, one being placed and stored at 3° to 5° C. until needed ("chilled pupae"), and the other being maintained at room temperature where, as previously described, diapause persists for at least five months ("diapausing pupae").

In a number of experiments related species of saturniid pupae were used; namely, *Samia walkeri*, *Callosamia promethea*, and *Telea polyphemus*.

The most important factor facilitating the investigation was the discovery of a method of continuous anesthesia for insects during operative procedures. This method, utilizing carbon dioxide and described by Williams (1946), permitted extensive and prolonged surgical manipulations without any loss of blood or apparent damage to the pupae. Other procedures will be described as encountered in the following discussion.

PARABIOTIC EXPERIMENTS

We have noted that diapausing pupae, after a period of exposure to low temperature, are rendered competent to develop when returned to room temperature, whereas, in contrast, pupae not subjected to chilling remain in diapause for at least five months. With these two types of animals at hand one is therefore in a position to test the fundamental nature of diapause by simply grafting one to the other so that they share the same blood. If diapause results from the *presence* of some factor inhibiting development, then such parabiotic combinations should fail to develop by virtue of the diapausing pupa distributing this inhibitory factor to the chilled individual. To the contrary, if diapause results from the *absence* of some necessary growth factor, both animals should develop, provided the chilled individual can supply double the minimal amount needed by a single animal.

In making these preparations a disc of pupal cuticle plus underlying hypodermis was cut from each pupa and the two animals placed together and held thus by the application of melted paraffin around the site. Most of the pupae were joined at the thoracic tergum (Figs. 1 and 3), but occasionally the junction was accomplished at the head or at the tip of the abdomen. Provided that the underlying heart is not injured and that no bubbles of air are trapped in either animal, such combinations are easily established and a high percentage survive.

In order to demonstrate that the operation in itself is without effects on dormancy, a series of ten diapausing pupae were successfully grafted to diapausing partners. Diapause persisted in each of these animals, adult moths being produced only after a minimum of 5½ months, the usual minimum length of time necessary for the spontaneous termination of diapause at 25° C. To the contrary, when diapausing pupae were joined to previously chilled individuals, the diapause in all viable preparations was terminated. In a series of 15 such combinations the pairs emerged as fully formed, active moths in an average of 41 days. Metamorphosis was complete both externally and internally, the only defect being a failure of the wings to expand after emergence (Fig. 2). This activation was not species- or, indeed, genus-specific, for it was possible to terminate the diapause of *Platysamia cecropia* by joining them to previously chilled pupae of *Telea polyphemus* (Figs. 3 and 4). Furthermore, sexual differences were without significance, for male pupae had the capacity to induce development of females, and vice versa.

A striking feature of all these parabiotic combinations is the fact that the animals invariably grow together so as to be connected by a pedicle, a phenomenon first noted by Crampton (1899) and later by Wigglesworth (1936) and Bodenstein (1938) in grafting procedures on insects. We shall have occasion subsequently to consider this union more fully, but in the present analysis suffice it to say that the

epithelial pedicle becomes externally chitinized and by way of its lumen permits a circulation of blood between the two animals.

In the earlier preparations the blood of the diapausing and chilled pupae in parabiosis was daily propelled to and fro by pressing accordion-like on the abdomen of each pupa alternately. This was later found to be wholly unnecessary, since development begins just as promptly without such forced mixing. It may also be noted that the completion of adult formation in the previously chilled animal occurs about $1\frac{1}{2}$ days earlier than in the diapausing partner (Fig. 5). This results from a corresponding delay in the initiation of development of the diapausing pupa. At all stages in adult differentiation the chilled pupa is therefore approximately $1\frac{1}{2}$ days in advance of the diapausing partner.

Thus these initial parabiotic experiments indicate some of the essential features of diapause. In general, they support the proposition that diapause results from the absence of a non-species-specific growth factor that is able to pass in parabiotic preparations from the activated to the dormant individual and evoke the initiation of adult development in the latter also.

BRAIN IMPLANTATION INTO DIAPAUSING PUPAE

If the termination of diapause is, indeed, accomplished by the action within the previously chilled pupa of a factor necessary for adult development, then it should be possible to demonstrate the organ in which this factor arises. For this purpose, various tissues and organs were removed from chilled pupae and implanted singly into diapausing individuals. When experiments of this sort were carried out, it was found that only one organ in the chilled pupa has the power to evoke development of diapausing pupae and that this organ is the brain itself. When the brain is removed from a chilled pupa and implanted into the head, thorax, or abdomen of a diapausing pupa, the latter is invariably induced to undergo adult development. Furthermore, *Platysamia cecropia* can be activated by the brains of *Samia walkeri*, *Callosamia promethea*, or *Telega polyphemus*—and, in fact, as far as the termination of diapause is concerned, there is a lack of species- and genus-specificity of brains among all these Lepidoptera tested. No other organ in the chilled pupa apparently possesses this power.

This effect of chilled brains is in marked contrast to that of diapausing brains,

EXPLANATION OF PLATE I

Approximately Life Size

FIGURE 1. Brainless, diapausing pupa of *P. cecropia* grafted to a chilled pupa of the same species.

FIGURE 2. Animals in Figure 1, after adult formation. The two insects have grown together and developed essentially simultaneously.

FIGURE 3. Brainless, diapausing pupa of *T. polyphemus* grafted to a chilled pupa of *P. cecropia*.

FIGURE 4. Animals in Figure 3, after adult development.

FIGURE 5. Parabiosis between two pupae of *P. cecropia*, removed from pupal cuticle before the completion of adult development. Development of the chilled pupa is $1\frac{1}{2}$ days in advance of that of the brainless, diapausing pupa.

FIGURE 6. Adult *Cecropia* moth produced by a brainless, diapausing *Cecropia* pupa, whose diapause was terminated by implantation of a brain from a chilled *Polyphemus* pupa.

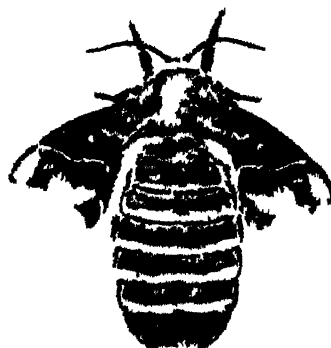
PLATE I



3



4



for the latter fail to terminate dormancy even when as many as eight are implanted into a single pupa.

The lack of species-specificity of implanted, chilled brains suggested the possibility that the effect might, conceivably, be mediated by an activation of the endogenous brain of the diapausing, "host" pupa itself. For this reason the vast majority of subsequent experiments were carried out on diapausing pupae from which the brains had been removed.

REMOVAL OF BRAIN

The operation devised for this purpose is easily performed, as follows. The insect, anesthetized with carbon dioxide, is placed on its back and by means of a sharp scalpel a rectangular window of pupal cuticle excised from its face. The underlying, semi-transparent hypodermis is thus exposed. With microscissors the latter is trimmed away, along with the tracheae traversing the operative field. In this procedure the frontal ganglion is frequently removed, but this has been found to be inconsequential. The brain now lies exposed and can be floated up toward the operator by pressing the pupal abdomen so that further dissection is performed in the pupal blood. With finely ground jeweler's forceps the nerves passing laterally to the site of the future adult eyes are grasped and broken. In similar fashion each brain hemisphere is carefully broken loose, in turn, from its tracheal supply, from the nerve passing posteriorly to the corpus allatum and corpus cardiacum and from the circum-esophageal connective. The brain can then be lifted free and examined in insect Ringer's solution.¹ Taking care to exclude all bubbles, the defect in the pupal chitin is then capped over with a small rectangle of thin, transparent plastic (cut from a plastic cover slip), which is sealed in place with melted paraffin.

The operation was originally performed with due regard to surgical asepsis; this was later found to be of little importance since the pupae are apparently not affected by the usual contaminating organisms. The mortality from the procedure is low and one ends up with a brainless pupa possessing a transparent window at its anterior end.

The defect in the hypodermis is rapidly repaired by a deposit of blood cells, followed by an ingrowth of cells and of tracheoles along the under surface of the plastic slip. Simultaneously, an intervening, delicate, transparent, "chitinous" lamella is elaborated. These relations permit a detailed study of the behavior of the hypodermis beneath the window, and, by means of an Ultrapaque, cellular activity has been followed under the oil immersion objective. It may be noted that this local process of repair occurs just as promptly in diapausing as in previously chilled pupae. Notwithstanding this fact, the process of repair is without overall effects on dormancy.

BRAIN IMPLANTATION INTO BRAINLESS DIAPAUSING PUPAE

The removal of the brain from diapausing pupae prior to using the animals experimentally proved to be an exceptionally significant maneuver. For whereas, as

¹ This physiological solution was originally devised by Ephrussi and Beadle (1936) for studies of *Drosophila*, but it works equally well for the *Lepidoptera* used in the present experiments. I am indebted to Dietrich Bodenstein for calling my attention to its composition, which is as follows: NaCl, 7.5 gm.; KCl, 0.35 gm.; and CaCl₂, 0.21 gm., per liter of water.

we have previously noted, diapausing pupae kept at 25° C. begin to escape spontaneously from diapause after about five months, no such activation occurs if the brain is removed. Among approximately 400 such pupae there has not been a single case of spontaneous development. It is therefore apparent that by removing the brain the pupa is maintained in permanent diapause. Such pupae remain alive for up to a year and finally die of dessication. Yet at any time during this period the diapause can be terminated by implanting into the brainless pupa the brain of a previously chilled animal (Fig. 6). Data in regard to a series of such pupae are given in Table I.

TABLE I
*Evocation of Adult Development of Brainless Diapausing Pupae
by Implantation of Brains from Chilled Pupae*

Species of host	Species of implanted brain	Number of experiments	Aver. time for adult emergence
<i>P. cecropia</i>	<i>P. cecropia</i>	16	35 days
<i>P. cecropia</i>	<i>T. polyphemus</i>	2	89
<i>P. cecropia</i>	<i>S. walkeri</i>	3	72
<i>P. cecropia</i>	<i>C. promethea</i>	2	63
<i>T. polyphemus</i>	<i>T. polyphemus</i>	2	64
<i>T. polyphemus</i>	<i>S. walkeri</i>	2	28

Manifestly, these experiments demonstrate that the termination of diapause requires the presence of an activated brain, in the absence of which adult development of these insects has not been observed. The conclusion is also self-evident that the termination of dormancy after diapausing pupae have been chilled results from the action of low temperature in rendering the brain able to evoke adult development. The other tissues in the diapausing pupa do not require such exposure to cold, for they are rapidly activated by implanting a brain which, alone, has been chilled.

This fact can also be readily demonstrated by removing strips of integument from diapausing pupae and implanting them into previously chilled pupae. Such diapausing tissues develop simultaneously with the host: the pupal cuticle is delaminated and a normally chitinized, adult cuticle, complete with scales and hairs, is found in the implant, in exactly the same fashion as described by Piepho (1938a and b) and Kühn and Piepho (1940) in studies of other aspects of insect metamorphosis. Thus the effect of low temperatures in facilitating escape from diapause can be explained solely on the basis of its effect on the brain.

PARABIOTIC EXPERIMENTS ON BRAINLESS DIAPAUSING PUPAE

As soon as the brain was definitely shown to be the source of the factor terminating diapause, ten more parabiotic combinations were prepared, but this time uniting *brainless* diapausing pupae with chilled individuals. Identical results were obtained: the two pupae in each combination grew together by a chitinized, epithelial, blood-filled pedicle and after an average of 44 days emerged as normal, active, adult moths.

BRAIN REMOVAL FROM PREVIOUSLY CHILLED PUPAE

Further information concerning the action of the brain in terminating diapause can be gained from a consideration of the behavior of chilled pupae. We have previ-

ously noted that these animals undergo no apparent development as long as they are maintained at the low temperature, but that within 1 to 1½ months after return to 25° C. the adult moth has fully formed and emerges. It is therefore worthy of note that if the brain of such chilled pupae is removed as soon as the insect is returned to the warm temperature, adult development never occurs and, in the same fashion as described for brainless diapausing pupae, dormancy persists indefinitely until the animal finally dies of dessication. Yet development can at any time be evoked by merely implanting into the head, thorax, or abdomen a brain obtained from another chilled pupa.

This phenomenon was studied more fully as follows. A series of thirty previously chilled pupae was placed at 25° C. and every few days the brains from several of these insects were removed and implanted into brainless diapausing pupae. The results may be summarized most briefly by saying that when the brain is removed within approximately the first 11 days, the brainless donors never show any development; such brains, in turn, evoke the development of brainless, diapausing pupae. In contrast, if the brain is removed from previously chilled pupae after approximately 17 days at 25° C., development continues to produce normal, brainless adults and the removed brains are without effect in terminating the dormancy of brainless, diapausing pupae.

These experiments have been repeated on a large scale, with special attention to the effect of brain removal during the critical period of 11 to 17 days. These more detailed studies were facilitated by establishing, at the outset, a transparent, plastic, facial window in each chilled pupa so that the behavior of the underlying hypodermis could be observed. It was at once apparent that the critical period, 11 to 17 days, was, in a sense, a statistical artifact, since, during this period, each individual achieves threshold activation during an extremely short interval, not exceeding a few hours. The actual critical period for each pupa is signaled by the initiation of hypodermal retraction from the overlying, facial chitin. Prior to this point, removal of the brain prevents development, and such brains evoke the development of brainless, diapausing pupae after an additional latent period of approximately three weeks. The moment hypodermal retraction is initiated, the brain can be dispensed with and such brains, when tested, are inactive.

Thus it is apparent that diapause persists even in chilled pupae until the latter have been exposed to a developmental temperature for an average of two weeks. Consequently, the activation of the pupal brain during exposure to low temperature must be conceived in terms of some physical or chemical alteration in the brain substance whereby the latter is rendered competent to produce or release its stimulating factor during subsequent exposure to a developmental temperature. The brain's action is then exerted and, thereafter, metamorphosis can proceed independent of its further participation.

ROLE OF THE CORPORA ALLATA

All the evidence so far considered reveals the brain as the organ of paramount importance in engendering and terminating diapause. Thus diapause in these species appears to result from the absence of a factor necessary for adult development, rather than from the presence of an inhibitory factor. The possibility remained, however, that the failure of the brain to exert its effect and the consequent onset

of diapause might, in turn, be due to inhibition arising elsewhere in the organism. The corpora allata were deemed the most likely source of such hypothetical inhibition and for this reason their significance in the production of diapause was studied.

As originally demonstrated by Bounhiol (1938) and subsequently confirmed by Piepho (1940; 1941), the corpora allata of Lepidoptera specifically inhibit pupation during the larval instars and thus oppose the activation of the presumptive imaginal tissues. This finding seemed so significant that comparable experiments were carried out on the caterpillars of *Platysamia cecropia* and *Telega polyphemus*. Although the removal of the corpora allata from caterpillars is a difficult procedure, it was accomplished in a sufficient number of immature (fourth instar) larvae to demonstrate that precocious pupation, indeed, results therefrom, the usual fifth instar being omitted. Furthermore, there is convincing evidence that the function of the corpora allata in inhibiting the imaginal discs disappears during the final larval instar and pupation then ensues. These findings have been considered in some detail for, although they concern pupation rather than adult differentiation, it is easy to see the importance of demonstrating whether, in potentially diapausing insects, the corpora allata once again inhibit the imaginal tissues, or the brain, and thus participate in the induction of diapause.

A series of experiments was therefore performed in which the corpora allata were removed (by a frontal approach) from diapausing pupae. Such pupae² invariably continued to diapause normally, and the removed corpora allata when implanted into previously chilled pupae were without effect in inhibiting adult development. As many as six corpora allata have been implanted into a single chilled pupa without retarding metamorphosis.

Similar negative results were obtained in regard to all other organs studied as a possible source of some inhibitory factor. For example, the diapausing brain itself is without inhibitory properties, for as many as six such brains have been implanted into a single chilled pupa without producing diapause. This is also true for the subesophageal ganglion, thoracic ganglion, gonads, imaginal discs, and strips of integument.

Although it cannot be denied that inhibitory factors may play a role in the production of diapause, the sum total of available evidence offers nothing to support this proposition. The brain itself remains the key to the production and termination of diapause in the species studied.

DISCUSSION

The role of the brain in terminating diapause, demonstrated for the first time in the present investigation, can to advantage be compared with its other functions in insect metamorphosis. Thus, in the bug, *Rhodnius*, the brain is necessary for moulting (Wigglesworth, 1940) and in the Lepidoptera it is now well established that the brain is also required for pupation (Kopec, 1922; Caspari and Plagge, 1935;

² It may be noted that the allatadectomized pupae ultimately escaped from diapause after the usual minimum period of 5½ months at 25° C. The resulting moths were wholly normal in all respects and could be induced to mate and lay eggs, which, in turn, were fertile. These findings apparently deny a participation of the corpora allata in the egg production of these species, a function described for them in certain other Orders of insects (Wigglesworth, 1936; Pfeiffer, 1939).

Kühn and Piepho, 1936; Bounhiol, 1938; Piepho, 1938a; Plagge, 1938). A surprising fact is that a role of the brain in imaginal differentiation has been specifically denied by all of these investigators of lepidopteran metamorphosis. The important point is that this conclusion was, without exception, based on studies of continuous, non-diapausing development. For such insects there can be little doubt that adult formation ensues even though the brain is removed from mature caterpillars in the last instar (i.e., after the "critical period" for pupation). The existence of this striking difference between continuous and diapausing development has been pointed out previously (Williams, 1942).

In the present investigation we have seen that all the evidence supports the theory that diapause results from an interruption in the normal processes of adult development. This point of view suggests that the brain is also necessary for evoking adult development in non-diapausing pupae. In non-diapausing individuals the brain may be viewed as having achieved its full developmental function precociously prior to pupation, whereas, in potentially diapausing animals, the brain first controls pupation and then months later after pupation it controls adult formation.

The action of the brain in terminating diapause in these saturniid pupae poses an additional problem of even greater interest; namely, the nature of the factor arising in the brain which so spectacularly evokes in dormant tissues a veritable flood of cellular activity. This problem will be considered in a subsequent communication.

SUMMARY

1. The physiological control of pupal diapause has been studied on a total of approximately 1200 pupae of the giant silkworms, *Platysamia cecropia*, *Telea polyphemus*, *Samia walkeri*, and *Callosamia promethea*.

2. The dormancy of diapausing pupae can be terminated readily by grafting them to activated (previously chilled) pupae. The two animals in each parabiotic combination grow together and some factor necessary for adult development passes from the activated to the dormant animal so that both develop simultaneously. This factor is not species- or genus-specific.

3. By implantation experiments the source of the factor terminating diapause is shown to be the brain and in this function a lack of species- and genus-specificity of brains is demonstrated.

4. In these species the well-known action of low temperatures in facilitating escape from diapause results from the effect of cold in rendering the brain competent to terminate dormancy. Actual termination of dormancy is accomplished only after the previously chilled brain has been exposed to a developmental temperature for an average of two weeks. The earliest indications of adult development then become evident and the brain, thereafter, is no longer required for the completion of metamorphosis.

5. Therefore, the effect of low temperatures on the brain must consist in some physical or chemical alteration in its substance whereby the latter is rendered competent to produce or release an imaginal-differentiation factor after return to a developmental temperature.

6. No evidence was found to support the theory that diapause results from the

presence of inhibitory factors. In this regard, the functions of the corpora allata are considered in some detail.

7. It is concluded that diapause in these species results from an interruption in the normal processes of development by virtue of a failure of the brain to supply a non-species-specific factor necessary for adult differentiation. Diapause is terminated when this factor is provided.

8. The significance of the brain in the development of diapausing pupae is considered in relation to its other functions, as reported in the literature. Notwithstanding a certain amount of evidence to the contrary, it is probable that even in the absence of diapause the brain plays a vital role in adult formation.

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FEEDING OF OYSTERS IN RELATION TO TIDAL STAGES AND TO PERIODS OF LIGHT AND DARKNESS

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INTRODUCTION

The beds of the American oyster, *Ostrea virginica*, are usually situated in inshore areas where tidal currents are strong and their regularity is sharply defined. Because of an almost continuous flow of water generated by the tidal movements over the beds populated with oysters, these mollusks live under continuously changing conditions. There is no doubt that the environmental changes caused by the tides may in certain instances noticeably affect the behavior of the oysters. It has never been satisfactorily demonstrated, however, that some of the vital processes of oysters, such as feeding, are carried on more vigorously during certain stages of the tide.

A review of the literature on this subject shows that the only attempt to study the relation between the feeding activities of oysters and the stages of the tide was made by Nelson (1921) who came to the conclusion that "The times of complete cessation or of commencement of feeding shows a rather definite correlation with the stage of the tide." In his other article on the feeding habits of oysters Nelson (1923) states that in the case of these mollusks relatively little food is taken on the ebb tide. Even as late as 1938 Nelson still refers to his old observations emphasizing the relative inactivity of the oyster during the outgoing tide (Nelson, 1938). These conclusions were accepted without verification by some other investigators and were finally incorporated in the article on oysters in the Encyclopaedia Britannica where it is stated that "The American oyster does not feed late at night and in early morning, and relatively little on the ebb tide" (Orton, 1929). At present, therefore, such behavior of oysters is recognized as an established fact.

In our investigations of various aspects of the biology of oysters of Long Island Sound and its tributaries, especially of Milford Harbor, it was noticed that these mollusks fed actively on the ebb tide and also during darkness. The stomachs of the oysters removed from the water during such periods contained large quantities of food. It was also observed on numerous occasions in the day time that during the last stages of the ebb the oysters populating the shallow flats were wide open and apparently feeding because they were expelling true faeces. These observations suggested that the conclusions expressed by Nelson and Orton probably did not apply to the oysters of the Long Island Sound and Milford Harbor region. However, since our observations were only of an occasional nature, they could not be regarded as entirely reliable. Therefore, to ascertain the feeding habits of our oysters in relation to the tidal stages, and to the periods of light and darkness, a systematic series of experiments was conducted in the Summer and Autumn of 1945. The solution of the problem undertaken was approached by using several methods, each of which contributed toward the correct evaluation of the results of the others.

The authors wish to express their sincere appreciation and thanks to Miss Frances Tommers for her helpful participation in all the phases of this work and especially for the difficult analysis of numerous kymograph records obtained in the course of these studies.

OBSERVATIONS AND EXPERIMENTS

Stomach content

The first method consisted in examining the volume of the stomach content of the oysters collected during different stages of the incoming and outgoing tides. As a rule, the oysters examined were approximately 4 inches in length. The examination was made immediately after the specimens were taken out of the water. After opening an oyster an incision penetrating to the stomach was made through the body wall and the content of the stomach was collected by means of a fine pipette inserted through the incision.

At first it was intended to employ a volumetric method for determining the quantities of food found in the stomachs but, eventually, this method was found unsatisfactory because the bodies of the oysters, regardless of quite a uniformity of their shells, displayed considerable difference in their volume and size. Since small-bodied oysters possessed smaller stomachs and were probably incapable of ingesting quantities of food equal to those of larger individuals, serious errors could be introduced in the interpretation of the results. Therefore, another method, in which each oyster was considered individually, was decided upon. This method consisted in a direct evaluation of the relative quantity of food found in the oyster's stomach. Three classes, namely: "large," "small," and "absent," were established to designate the results of the examination. The oysters which were classified as containing large quantities of food could easily be recognized because, as soon as the incision was made in their bodies, the stomach content freely flowed out. This phenomenon is probably familiar to all investigators who worked with oysters. The animals placed in the second class were those whose stomachs contained only small quantities of food. The third class was composed of oysters with empty stomachs. The animals of the latter group were especially carefully examined to be certain that they contained no food.

The largest number of oysters examined in the course of these studies was collected from the cultivated oyster beds of Long Island Sound. This group, consisting of 1000 individuals, was composed of ten samples, each usually containing 100 oysters. These samples were collected at approximately weekly intervals during June, July, and August 1945. Each of the large samples consisted in turn of ten smaller ones composed of 8 to 12 individuals. The latter samples were dredged from our ten collecting stations established in the Sound. All these stations, located at a depth ranging from 10 to 30 feet at the mean low water stage, were subjected to strong tidal currents. The exact time of the collection of each sample was recorded and later correlated with the stage of the tide.

The collection of samples from all the stations extending along the shore for a distance of about 25 miles usually required from six to eight hours. Thus, it was sometimes possible to continue to collect samples during the entire period of the outgoing or incoming tide. The stations were not always attended in the same sequence to guarantee that the sampling was of a random nature.

The results of the examination of 1000 oysters offered rather conclusive evidence that the mollusks were feeding as actively on the ebb tide as they did during the flood (Table I). As a matter of fact, the per cent of oysters containing a large quantity of food was somewhat higher on the ebb than during the flood. Furthermore, only 6 per cent of the oysters collected during the ebbing tide possessed empty stomachs, while among the individuals dredged during the flood 10 per cent showed a complete absence of food. It is significant that among the oysters collected during the low water stage the per cent of the individuals containing a large quantity of food was higher, and that of the animals with empty stomachs lower than at many other stages

TABLE I

Relative quantities of food in stomachs of 1000 oysters collected at different tidal stages in Long Island Sound, June, July, and August 1945

Stage of tide	Oysters examined	Quantity of food			Per cent		
		Large	Small	Absent	Large	Small	Absent
Flood							
1st hour	41	31	6	4	75	15	10
2nd hour	60	51	2	7	85	3	12
3rd hour	85	58	9	18	68	11	21
4th hour	75	66	5	4	88	7	5
5th hour	85	72	8	5	85	9	6
High water	110	91	9	10	83	8	9
Total	456	369	39	48	81	9	10
Ebb							
1st hour	80	66	11	3	82	14	4
2nd hour	80	69	7	4	86	9	5
3rd hour	120	102	10	8	85	8	7
4th hour	90	76	10	4	85	11	4
5th hour	107	84	13	10	79	12	9
Low water	67	59	6	2	88	9	3
Total	544	456	57	31	84	10	6
Grand total	1000	825	96	79	82	10	8

of the tide. While this observation cannot be interpreted as definite proof that oysters feed most efficiently just prior and during the low water stage, it shows, nevertheless, that they do not cease, or noticeably decrease, their feeding activities during late ebb.

Additional observations on the relative quantities of food in the stomachs of the oysters at the end of the flood and during the entire period of ebb were made in Long Island Sound on August 21, 1945. On that day a station was chosen in 20 feet of water on one of the beds planted with 4-year-old mollusks. The location of the station was designated by a special buoy. The samples were collected at hourly intervals beginning one hour prior to the high water stage (Table II). Altogether eight samples, each composed of 20 oysters, were collected and examined.

The salinity of the water ranged from 27.31 p.p.t. soon after high water to 25.45 p.p.t. at low water.

The results of the examination again showed that in the majority of the oysters the stomachs contained large quantities of food during all stages of the ebb (Table II). The observations also indicated that there was no decrease in the number of oysters with food-filled stomachs parallel with the falling of the tide. On the contrary, the largest number of oysters containing large quantities of food was found only one hour prior to low water.

It is significant that among the oysters examined during the last three hours of the ebb not a single individual was found with an empty stomach. While the presence of food in the oysters dredged during the early stages of the outgoing tide could be possibly explained by assuming that this food was ingested during the last

TABLE II

Relative quantities of food in stomachs of 160 oysters collected at hourly intervals during last hour of flood and during ebb from a station established in 20 feet of water in Long Island Sound, August 21, 1945. Each sample composed of 20 oysters.

Stage of tide	Temperature °C.	Quantity of food		
		Large	Small	Absent
Flood				
5th hour	21.2	11	8	1
High water	20.9	17	3	0
Ebb				
1st hour	21.1	14	6	0
2nd hour	21.0	19	1	0
3rd hour	20.9	17	2	1
4th hour	21.1	19	1	0
5th hour	20.6	20	0	0
Low water	20.4	16	4	0
Total		133	25	2

hour of flood, such an explanation cannot be offered for the presence of food in the oysters examined from three to six hours after the high water stage. Our observations performed under laboratory conditions on oysters kept in water of 20.0° C. showed that these mollusks pass the particles of food through their entire digestive system from 1 hour 20 minutes to approximately 2 hours and 30 minutes. Therefore, it seems rather improbable that the food found in the oysters during the latter part of the ebb was that ingested during the late stage of the flood, four to six hours prior to examination.

In general, the observations made on 160 oysters on August 21, 1945, showed that the oysters of Long Island Sound fed very actively during the ebb, and that the relative quantities of food found in their stomachs during that period were at least equal to or even exceeding those recorded during the last hour of the preceding flood.

Although observations in Long Island Sound have demonstrated that there was no correlation between the stages of the tide and the quantities of food found in the

stomachs of the oysters, it was desirable to supplement the data already available with additional observations on the oysters living under ecological conditions rather different than those of Long Island Sound proper. An area in Milford Harbor near the dock used for laboratory needs, was chosen for these observations. A large number of oysters living on the bottom near the dock made such an arrangement especially convenient.

Milford Harbor was selected because it was a typical example of a small partially inclosed body of water where extensive natural beds had existed. In recent times many of the beds were destroyed by overfishing, and the profile of the bottom was markedly changed by the dredging of a wide and deep channel. Nevertheless, the oysters quickly reestablished themselves in more shallow sections of the Harbor, and at present are quite common. A good setting of oysters regularly occurs in the Harbor, indicating that the conditions are favorable for the propagation of these mollusks. Usually changes in the temperature and salinity of the Harbor water during the tidal cycle are more pronounced than in Long Island Sound proper, where both these factors remain very steady (Loosanoff and Engle, 1940).

The observations consisted in examining the stomach content of the oysters at hourly intervals throughout a 24-hour period. The samples, each composed of six individuals, were suspended one day prior to the beginning of the examination in baskets made of 2-inch mesh poultry wire. All the baskets were kept at the same depth, namely, one foot below the mean low water line. They were separated from each other by a distance of approximately one foot and, therefore, the removal of any of the baskets did not disturb the oysters of the other containers. The experiment continued from 7:30 A.M. of July 27 until 8:00 A.M. of July 28, covering three low and two high water stages, and including periods of light and darkness (Table III). During this period the temperature of the water ranged from 22.0 to 25.0° C., the salinity fluctuated between 22.68 and 28.44 parts per thousand, and the pH from 7.7 to 8.7.

The data obtained indicated that during the two flood and two ebb periods covered, the majority of the oysters contained large quantities of food. Of the total number of 150 oysters examined 86 per cent belonged to that group. This figure closely approached that for the Long Island Sound oysters where 82 per cent were found to possess large quantities of food (Table I). Only 4 per cent of the Milford Harbor oysters were found with empty stomachs, the figure being too low to suggest that large groups of the oyster population ceased feeding for appreciatively long intervals during the period of observation.

More detailed studies of the data given in Table III do not offer sharply defined evidence which would lead to the conclusion that the oysters collected during the flood contained more food than those collected at ebb, or vice versa. Although it is true that all but one oyster collected during the second flood period, extending from 8:40 P.M. to 1:58 A.M., contained large quantities of food, virtually the same observations were made during the preceding period of ebb when 32 out of 36 oysters showed full stomachs. In each case only one oyster with an empty stomach was found. If the condition of the oysters during the first flood period (8:30 A.M. to 1:30 P.M.) is compared with that of the oysters examined during the second ebb period (3:00 A.M. to 8:00 A.M.), it will be found that in each case 28 individuals showed large, and 6 showed small quantities of food, while 2 oysters were with empty stomachs.

TABLE III

Temperature, salinity and pH of the water, and relative quantities of food in stomachs of oysters examined at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945. Each sample of oysters consisted of six individuals.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food			Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent						Large	Small	Absent
Low water	7:30 A.M.	24.2	22.68	7.7	6	0	0								
Flood															
1st hour	8:30 A.M.	24.0	27.74	7.7	4	2	0	Flood	8:45 P.M.	24.5	24.40	8.7	6	0	0
2nd hour	9:30 A.M.	23.2	26.59	8.0	3	2	1	1st hour	9:45 P.M.	23.9	25.73	8.5	6	0	0
3rd hour	10:30 A.M.	23.3	27.65	8.0	6	0	0	2nd hour	10:45 P.M.	23.3	26.00	8.5	6	0	0
4th hour	11:30 A.M.	23.0	28.44	8.0	3	2	1	3rd hour	11:40 P.M.	23.3	26.00	8.5	5	0	1
5th hour	12:30 P.M.	23.1	27.65	8.1	6	0	0	4th hour	12:45 A.M.	23.1	26.54	8.5	6	0	0
High water	1:30 P.M.	23.1	26.33	8.2	6	0	0	5th hour	1:58 A.M.	23.0	26.27	8.5	6	0	0
Ebb								High water							
1st hour	2:30 P.M.	23.2	27.65	8.2	6	0	0	Ebb							
2nd hour	3:30 P.M.	23.0	28.51	8.3	6	0	0	1st hour	3:00 A.M.	23.0	26.00	8.5	5	0	1
3rd hour	4:30 P.M.	23.4	26.39	8.3	4	2	0	2nd hour	4:00 A.M.	23.1	26.27	8.5	6	0	0
4th hour	5:30 P.M.	23.9	26.13	8.3	5	0	1	3rd hour	5:00 A.M.	23.3	26.27	8.4	4	2	0
5th hour	6:30 P.M.	24.9	24.52	8.1	5	1	0	4th hour	6:00 A.M.	23.0	25.73	8.1	3	2	1
Low water	7:40 P.M.	24.7	24.13	8.4	6	0	0	5th hour	7:00 A.M.	23.2	25.47	8.1	5	1	0
								Low water	8:00 A.M.	23.4	25.47	8.0	5	1	0
Total													129	15	6
Per cent													86	10	1

Perhaps it should be emphasized that among the 18 animals collected at the three low water stages (Table III) all but one oyster had large quantities of food in their stomachs.

Studies of the same nature as those made on July 27 and 28 were performed again on August 9. However, in the latter case observations were made only during one flood and one ebb stage covering a period of about 12 hours. The results of the observations are incorporated in Table IV together with data on the water temperature, salinity and pH recorded at the collection of each sample. The results showed that more oysters with large quantities of food were found in the group collected

TABLE IV

Relative quantities of food in oysters examined at hourly intervals during a 12-hour period in Milford Harbor on August 9, 1945. Each sample composed of 6 oysters. Temperature, salinity and pH are indicated for each stage of the tide.

Stage of tide	Time of day	Temperature °C.	Salinity p.p.t.	pH	Quantity of food		
					Large	Small	Absent
Flood							
1st hour	7:55 A.M.	22.2	24.99	7.9	0	5	1
2nd hour	8:55 A.M.	21.9	25.72	7.9	1	5	0
3rd hour	9:55 A.M.	21.6	25.72	7.9	3	3	0
4th hour	10:55 A.M.	21.6	25.99	7.9	3	1	2
5th hour	11:55 A.M.	21.8	25.44	7.9	6	0	0
High water	12:55 P.M.	21.7	25.72	7.9	5	1	0
Total					18	15	3
Ebb							
1st hour	1:55 P.M.	22.0	25.72	8.0	4	2	0
2nd hour	2:55 P.M.	21.8	25.72	8.1	3	2	1
3rd hour	3:55 P.M.	22.0	25.72	8.1	6	0	0
4th hour	4:55 P.M.	23.9	25.44	8.1	6	0	0
5th hour	5:55 P.M.	24.4	23.84	8.0	4	2	0
Low water	7:05 P.M.	25.1	21.44	8.0	6	0	0
Total					29	6	1

during the ebb than among the animals examined during the flood. However, no significant difference was noted between the two groups in the number of oysters with empty stomachs.

In summarizing all our observations on the relative quantities of food present in the stomachs of the oysters during different tidal stages the conclusion may be formed that, as far as ingestion of food is concerned, the oysters of Long Island Sound and Milford Harbor do not show a definite preference either to ebb or to flood. During either stage the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally. The data obtained fail to lend any support to the unqualified opinion that the American oyster, *O. virginica*, takes relatively little food on the ebb tide.

Rate of water pumping in relation to tide

To determine the rate of water pumping of oysters and, therefore, the efficiency of their feeding at different stages of the tide an apparatus was constructed which permitted the measurement of the actual quantities of water passing through an oyster (Fig. 1). This apparatus was installed on the edge of the dock situated along the western shore of Milford Harbor where a swift tidal current flowed unobstruct-

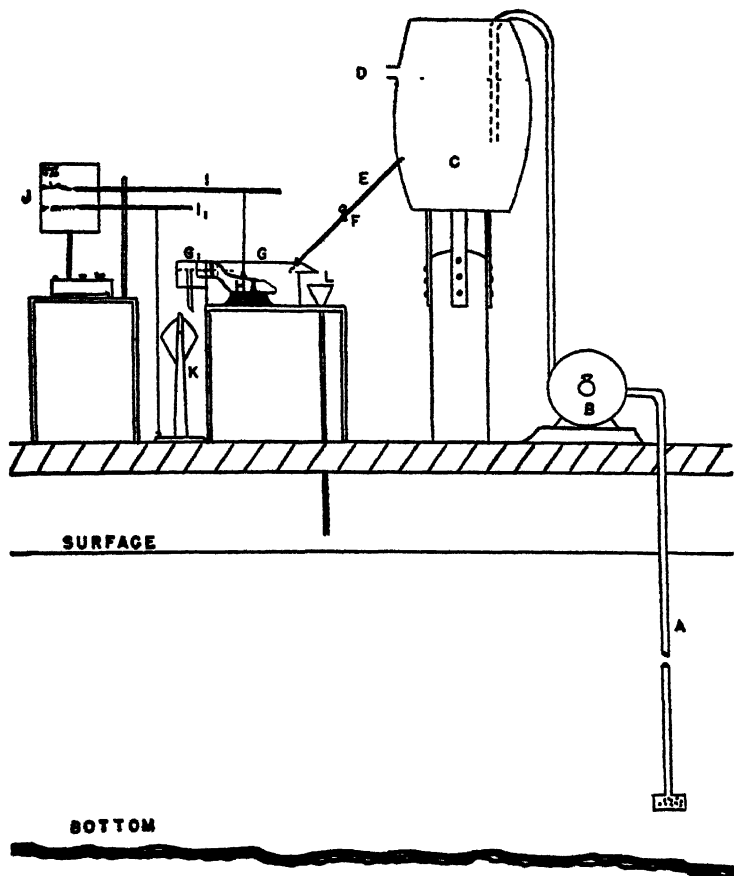


FIGURE 1. Diagram of the apparatus used in determining the quantities of water pumped by the experimental oysters. Description in text.

edly. The water supply was obtained through the hose, *A*, being pumped by the pump, *B*, into the storage barrel, *C*. The intake end of the hose, protected by a screen of large mesh always remained in the same position, six inches above the bottom. A constant water level in the barrel, *C*, was maintained by allowing the excess water to escape through the overflow outlet, *D*, to which a length of hose was attached. The capacity of the pump was such that the water in the storage barrel

was renewed every 4 or 5 minutes. Thus, the experimental oysters were receiving water, which was changing parallel with the changes of the tide.

The water was fed to the experimental oysters through the tube, *E*, provided with a flow-adjusting cock, *F*. The constant level oyster chamber, *G*, contained the oyster, *H*, the excurrent side of which was covered with a rubber, cone-shaped apron which conducted the water pumped by the oyster into the smaller chamber, *G*₁. Moore (1908) and Nelson (1936) were the first to apply the rubber apron, while Galtsoff (1926) devised and began to use the chamber. A string glued to the upper shell of the oyster was attached to the counter-balanced lever, *I*, which recorded every movement of the shell on the kymograph, *J*.

The water pumped by the oyster into the chamber, *G*₁, overflowed through the glass tube standpipe into the tripping vessel, *K*, of known capacity. When the vessel was filled with the water pumped by the oyster it tripped over, emptying its contents, and at the same time striking a string attached to the lever, *I*₁, which made a mark on the kymograph, *J*. Thus, each tripping was recorded, and because the capacity of the vessel was known, the quantity of water pumped by the oyster during certain intervals could be ascertained easily. The excess water entering the chamber, *G*, but not utilized by the oyster flowed out through the outlet, *L*. The part of the experimental apparatus containing the oyster chambers and the kymograph was kept in a small shed to protect the oyster from the effects of the sun and from possible disturbances caused by the wind.

The oysters were placed in the apparatus usually from one to two hours before high or low water. This time was allotted to the oysters to open their shells and to begin pumping water at a normal rate. This introductory period was not included in the analysis of the pumping activities of the oysters.

At the end of the introductory period, which usually coincided with the high or low water stage, observations were carried on for 12 or 13 hours, covering one ebb and one flood stage. In this manner records of 27 oysters were obtained. Fortunately, in almost all cases the oysters remained open continuously throughout the period of exposure. The experiments were conducted at temperatures ranging from 19.1 to 25.8° C., a range considered very favorable for the pumping activities of oysters (Galtsoff, 1928).

In analyzing the kymograph records obtained in the course of these studies it was found convenient to divide each flood or ebb period into six hourly subperiods. However, since the ebb and flood periods of Milford Harbor are not usually of exactly 6-hour duration, the data for the last hour of each stage had to be arrived at by determining the quantity of water pumped by the oyster from the end of the fifth hour until the change of the tide and then calculated for a 60-minute period.

Analysis of the data showed that, in general, the rate of pumping of the oysters during the flood stages was somewhat slower than that during the ebbing periods (Table V). However, because of significant differences in the rate of pumping shown by individual oysters within the same hour of a tidal period, and after considering all the aspects of the data secured the opinion was formed that the oysters of Milford Harbor feed actively at all stages of the tide, and that the rate of feeding during the ebb is at least equal to and sometimes may be even more rapid than during the flood.

In Milford Harbor the strongest tidal currents occur in the middle of the period between the high and low water stages. This period, therefore, corresponds to the

third and fourth hours of each stage. There is no evidence, nevertheless, that during this period the pumping of the oysters was more energetic than during the preceding or successive periods (Table V). It is of interest to note, however, that during the two last hours of the ebb the rate of pumping was somewhat accelerated.

The rapid rate at which many of the experimental oysters pumped water during ebb is well demonstrated in the photograph of the kymograph record showing the rate of pumping and shell movement of one of the experimental animals (Fig. 2). The period of observation lasted from 9:34 A.M. until 10:13 P.M., September 12, covering one complete flood and ebb period. Each vertical line of the lower record was made by the tripping vessel (Fig. 1), the capacity of which was 255 cc. Examination of the lower part of the record, which, incidentally, was made with the help of a dissecting microscope, a method always employed when the pumping was rapid and the marks on the kymograph were made close to each other, provided the following information: the minimum quantity of water pumped by the oyster during a single hourly period of the flood was 4080 cc., and the maximum, 20,655 cc. The

TABLE V

Mean of quantities of water (in cubic centimeters) pumped by oysters during each hour of the flood and ebb periods. The data are based on the kymograph records of 27 oysters, Milford Harbor, Summer of 1945.

Stage of tide	Mean	Stage of tide	Mean
Flood		Ebb	
1st hour	15,952±1,214	1st hour	15,384± 787
2nd hour	14,411±1,578	2nd hour	15,962± 830
3rd hour	12,875±1,427	3rd hour	16,186±1,021
4th hour	12,470±1,389	4th hour	17,458±1,195
5th hour	13,438±1,130	5th hour	17,916±1,063
6th hour	14,131±1,040	6th hour	17,577± 944

average hourly rate of pumping for the entire flood period was 13,260 cc. For the ebb period these figures were 15,810, 23,715, and 20,244 cc. respectively.

Examination of other records of the same series revealed that during the ebb some of the oysters averaged from 25 to 27 thousand cc. per hour, while the maximum rate of pumping in some instances ranged from 31 to 34 thousand cc. per hour. Having in possession a large number of kymograph records of this nature one may well be inclined to disagree with the opinion that the American oyster is relatively inactive during the outgoing stage of the tide.

In connection with this discussion an interesting deduction can be made concerning the efficiency of the pumping mechanism of oysters. The average weight of the oyster meat removed from the shell the length of which is 4 inches is approximately 20 grams and its volume is usually not more than 20 cc. This organism, nevertheless, is capable of pumping 30 thousand cc. or more of sea water per hour. In other words, the volume of water passing through the oyster gills in one hour may be more than 1500 times greater than the volume of the oyster's body, a fact well attesting the efficacy of the feeding mechanism of this mollusk.

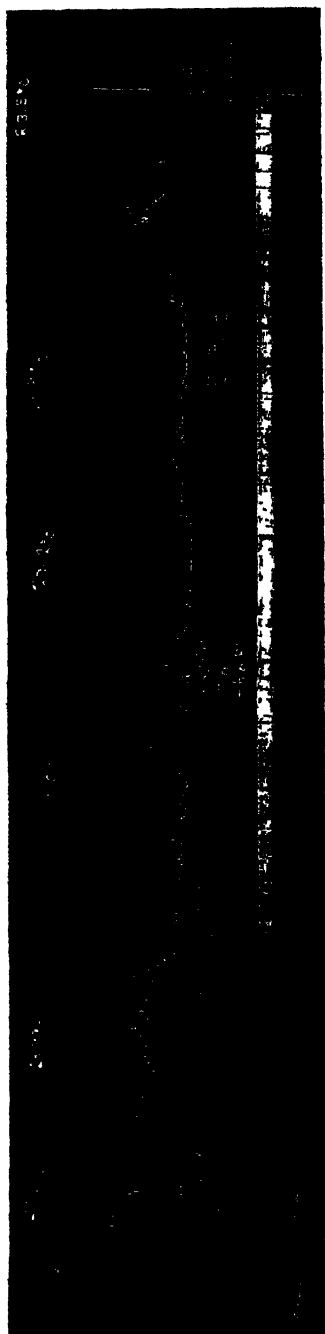


FIGURE 2. Photograph of kymograph record showing the shell movements (upper line) and rate of pumping (lower line) of oyster no. 19 obtained during the flood and ebb stages in Milford Harbor on September 12, 1945. Each vertical line of the lower record designates emptying of the tripping vessel (Fig. 1) of 255 cc. capacity. *L.*, *W.*, and *H.*, indicate the low water and high water stages respectively.

Opening and closing of shells in relation to tide

Experiments for determining the presence or lack of correlation between the opening and closing of the oyster shells in relation to the tidal stages were also conducted in the Summer of 1945. The method used was the same as that successfully employed in the studies of the shell movements of hard shell clams, *Venus mercenaria*, and of the edible mussel, *Mytilus edulis* (Loosanoff, 1939, 1942). A description of this apparatus has already been published in full (Loosanoff, 1939) and, therefore, need not be repeated. It will be sufficient to say that this apparatus permitted registering on the Foxboro recorder of every shell movement of oysters kept under virtually natural conditions on the platform installed on the small oyster bed existing on the bottom of Milford Harbor. Even at low tides the experimental oysters were covered with at least 2 feet of water.

The area where the apparatus was installed was subjected to strong tidal currents which, during the spring tides, attained a velocity of 1.2 feet per second on the flood, and 1.5 feet per second on the ebb. During the neap tides, however, the velocity of the flood current was only 0.8 foot per second, and that of the ebb current, 1.3 feet per second. The mean range of tide in Milford Harbor is 6.6 feet, but during the spring tides high water occasionally reaches the 9-foot mark. In Milford Harbor, the same as in Long Island Sound, the strongest tidal currents occur in the middle of the period between high and low water. The slack usually coincides with the time of the high and low water stages.

Usually the records of two oysters were taken simultaneously—each record covering a 24-hour period. The temperature of the water was also continuously registered by Brown's recording thermometer which was installed near the shell movement recording apparatus, the bulb of the thermometer being only 5 inches away from the experimental oysters. The temperature range extended from 17.0 to 28.0° C., averaging approximately 22.0° C. Altogether 64 records of 18 different oysters were obtained in the course of these observations.

That the conditions in the Harbor were favorable for the existence of the mollusks was well shown by the growth of the young oysters which attached themselves to the concrete base of our apparatus used for studying the shell movements of the experimental animals (Fig. 3). The attachment, or, as it is more commonly called, the setting of the oysters took place early in August, soon after the apparatus was first placed in the water. The examination made late in September showed that the young oysters grew very well, regardless of the fact that the base was often removed from the water for short periods to change the experimental mollusks. This rapid growth of the young oysters, as well as their generally good condition, indicated that the experimental animals, the shell movements of which were recorded, were also subjected to favorable environment.

Analysis of 64 complete records showed that on an average the oysters remained open 22 hours and 39 minutes, or 94.3 per cent, and closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period. Our figure for the duration of openness of the oyster shells is quite close to that of Nelson (1921) who, basing his conclusions on the records of 3 oysters, found that these animals remained open on an average of 20 hours per day, but is much higher than that offered by Galtsoff (1928) who found that the average period the oysters remain open is 17 hours and 7 minutes per day. However, the difference between Galtsoff's figures and ours may be explained on

the basis that his observations were made on the oysters placed in the aquarium, while our animals were kept on the bottom of the Harbor, where conditions were different from those of the laboratory. Nevertheless, Galtsoff expresses the opinion, which coincides with ours, that oysters have a tendency to keep the shells open as long as possible.

Further statistical studies of the data collected showed that during the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the

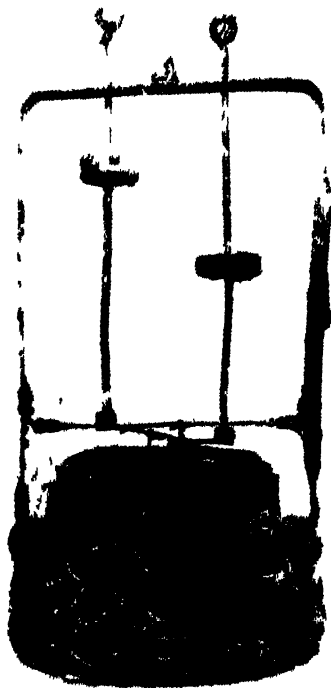


FIGURE 3. Photograph of the base of the instrument employed to record the shell movements of the oysters kept on the bottom of Milford Harbor. Note the healthy and vigorously growing young oysters attached to the sides of the base. Large experimental oysters imbedded in small concrete blocks can be seen on the top of the base.

time, whereas during the ebb periods the shells were open 95.2 per cent. Obviously, the difference between the two figures is rather insignificant, indicating that the behavior of the oysters is quite similar during the opposite stages of the tide.

Another trait of similar behavior of the oyster during the two different tidal stages was indicated by the fact that almost an equal number of records showing that the animals kept their shells open 100 per cent of the time was obtained for each stage. Thirty-three such records were made during the flood periods, and 32, during the ebb

Feeding in relation to time of day

While conducting these studies the opportunity presented itself to verify the statements that the feeding of oysters is considerably slowed down (Nelson, 1921, 1923) or entirely ceases (Orton, 1929) at night and in the early morning. The material for studying this problem was available from all three phases of our investigation, namely, observations on the stomach content of oysters, rate of pumping, and opening and closing of the shells.

Examination of the stomach content of the oysters made at hourly intervals during a 24-hour period in Milford Harbor on July 27 and 28, 1945, has shown that during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. (E.W.T.), the oysters contained as much food as during the hours of light (Table III). During the largest part of the period of darkness, until 5:00 A.M., almost all the oysters contained large quantities of food. The percentage of individuals with full stomachs during this period would compare favorably with that of the animals examined during the daytime. Numerous other examinations of the stomach content of the Milford Harbor oysters made between 1:00 and 6:00 A.M. during the Summer of 1945 also showed that the majority of the oysters had full stomachs. Obviously, these observations do not offer any support to the conclusion that oysters do not feed at night.

The results of the experiments in which the rate of pumping of the oysters was determined also contradict the above mentioned conclusion. Observations performed at night showed that the oysters remained active and fed very vigorously. The results of the experiment conducted during the night of July 27-28 were especially significant because in that case the period of late night coincided with the ebb (Fig. 4). Thus, the effects of two presumably unfavorable factors were combined. Nevertheless, as is shown in Figure 4, both experimental oysters remained open and continued to pump water at a very rapid rate during the entire period of darkness. When one of the animals closed at about 6:30 A.M. it already was one hour after sunrise. During the period of darkness the average rate of pumping of oyster no. 92 (upper record) was 17,934 cc. per hour, and the maximum, 19,272 cc. per hour. For oyster no. 98 (lower record) these figures were 24,622 and 30,600 cc. respectively.

Because this experiment was of a 24-hour duration data were also available on the rate of pumping of the same two oysters in daylight. The average rate of pumping of oyster no. 92 during that period was found to be 12,684 cc. per hour, and the maximum, 19,053 cc. per hour. For the second oysters the corresponding figures were 24,565 and 31,875 cc. Thus, in this case, the same as in the case of studies of the stomach content of the oysters, the conclusion may be offered that oysters feed actively during darkness. Furthermore, the average rate of pumping at night was not lower than during the daytime.

A series of supplementary experiments on the effect of light and darkness upon the rate of pumping also failed to show a significant difference in the behavior of oysters. These experiments were conducted in a dark room, where an apparatus of the type shown in Figure 1 was installed. Three or four oysters were tested simultaneously. During the period of light the oysters were illuminated with small floodlights. The intensity of light at the surface of the oysters, as determined by the Weston photronic exposure meter, was in excess of 1000 candles per square foot.

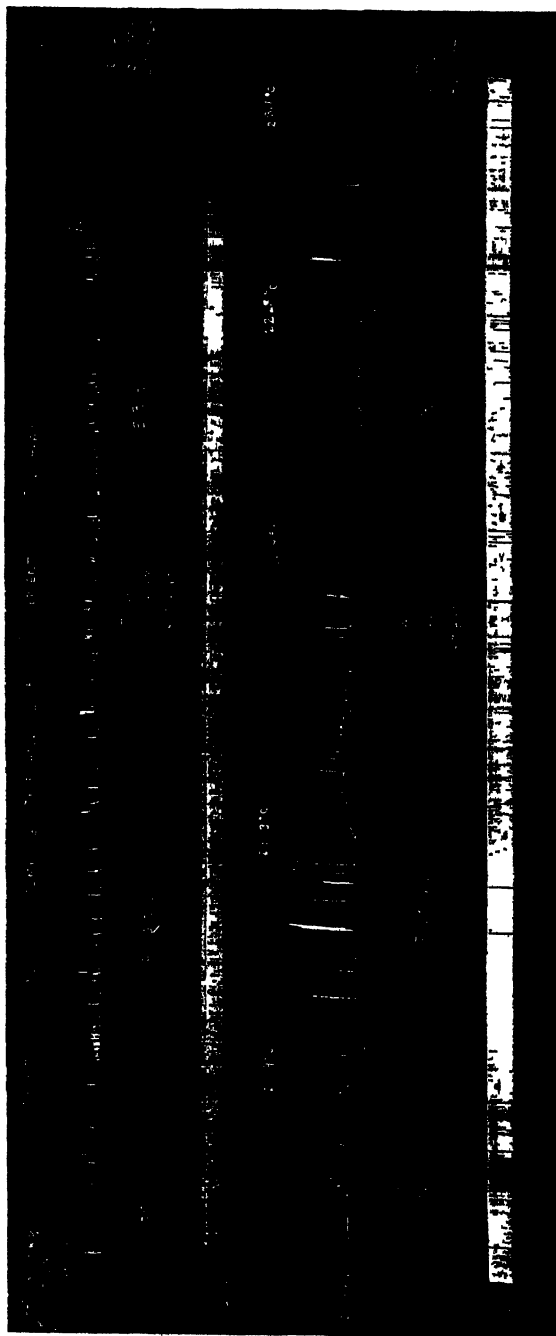


FIGURE 4. Photograph of the kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters during the period extending from 7:50 P.M., July 27 until 8:17 A.M., July 28, 1945. Each vertical mark of the second line designates emptying of the tripping vessel of 219 cc. capacity, while each mark of the fourth line shows the dumping of 255 cc of water. The records show that both oysters fed very actively during the period of darkness, which extended from 8:33 P.M. until 5:33 A.M. L. W. and H. W. indicate the low water and high water stages respectively.

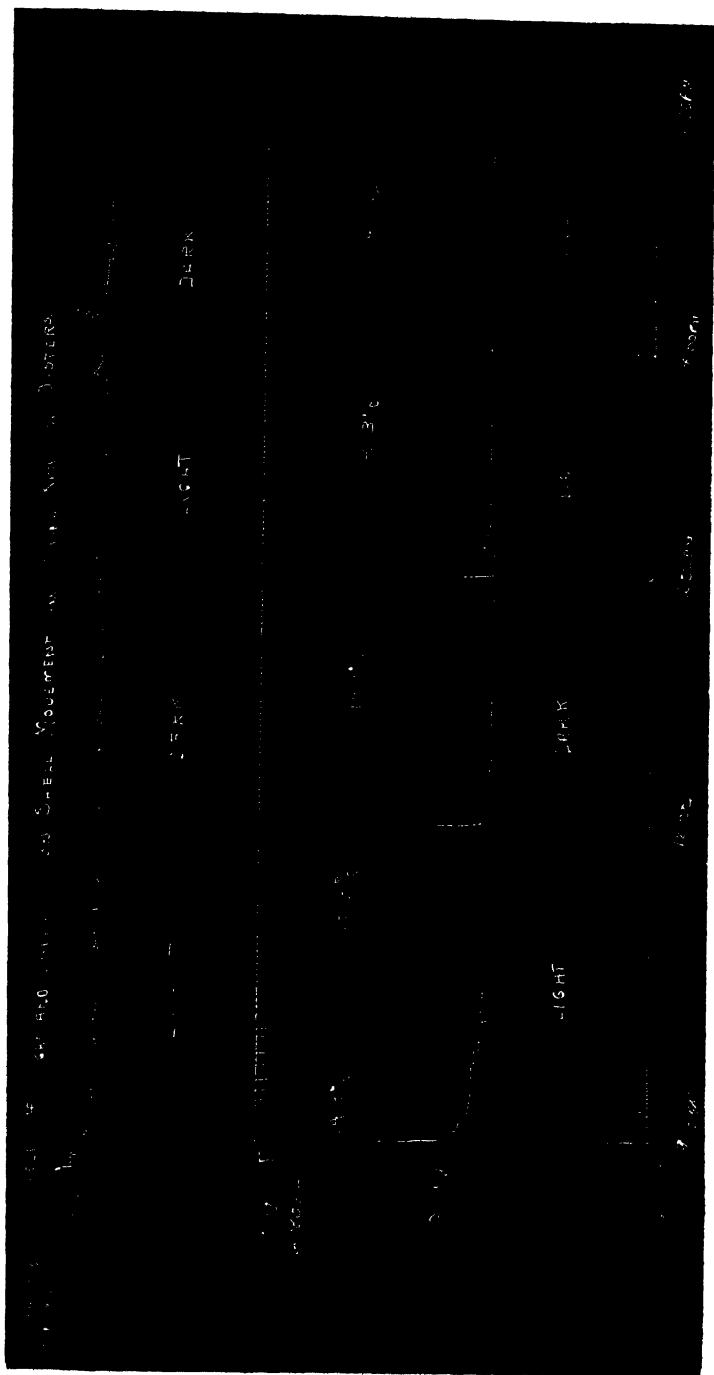


FIGURE 5. Photograph of kymograph record showing the shell movements (1st and 3rd lines) and the rate of pumping (2nd and 4th lines) of two oysters exposed to alternating periods of light and darkness. Each vertical mark of the second line designates the emptying of the tripping vessel of 280 cc. capacity, while each mark of the fourth line shows the dumping of 237 cc. of water.

The oysters were kept in the apparatus for approximately 9 hours (Fig. 5). During this time the mollusks were exposed to two periods of light and two of darkness. However, the initial period, of approximately 3 hours, was not included in the analysis, as this time was given to the oysters to become accustomed to the experimental condition. To equalize the data half of the experiments began with a period of light, and the other with that of darkness. Altogether 30 records of 10 different oysters were obtained. Analysis of the data showed that the mean hourly rate of pumping during the periods of light was 5350 ± 445 cc. For the periods of darkness this figure was 5036 ± 385 . Thus, the rate of pumping during periods of light closely resembles that during periods of darkness.

Finally, the data on the same subject were taken from our 64 daily records of the shell movements of oysters. Analysis of these data showed that on an average the shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the periods of darkness. The difference of less than one per cent is not considered as significant in this case, and, therefore, it may be concluded that in our experiments no correlation was found between the periods of closure of the shells and darkness. These results are in agreement with those of Galtsoff (1928) who in his article disagrees with Nelson's (1921, 1923) conclusion that the period of darkness, between 11:00 P.M. and 4:30 A.M., should be considered as a time of rest for oysters.

DISCUSSION

The advocates of the opinion that oysters considerably reduce their feeding activities during the periods of ebb failed to suggest in their publications any factors which could be considered as responsible for the change in the behavior of the mollusks. It could be easily understood that in some areas, where the periods preceding and coinciding with the low water stage are accompanied by distinctly unfavorable changes in the environment, the oysters would temporarily slow down or even cease feeding. For example, a sharp decrease in salinity could compel the oysters to be relatively inactive. However, according to Nelson (1921) the reduction in salinity was not the cause. This conclusion is based upon his statement that in Huey's Creek, where the experiments were conducted, the periods of complete cessation or of the commencement of feeding, although showing a definite correlation with the stage of the tide, occurred independently of the changes in the density of the water, because such changes were usually of small magnitude.

Changes in the turbidity and temperature of the water were also considered as unimportant by Nelson (1923), who concluded that "The rate of filtration of water during any given period of time, as deducted from the rapidity and extent of ejections of accumulated sediment from the mantle cavity, may vary widely independently of the temperature and the turbidity of the water." In the same article Nelson also stated that "No correlation could be shown between the food content of the water and the periods of inactivity of the oyster." All these conclusions were based upon the experimental data first reported by Nelson in 1921. Thus, according to that author, neither changes in salinity or temperature, nor changes in the turbidity or quantity of food present in the water affected the rate of feeding of oysters. Yet, because of some undetermined factors these mollusks fed much less actively during the ebb stages.

In discussing Nelson's (1921) work it is necessary to mention that his experiments were devised to study the shell movements of the oysters, but not the rate at which these mollusks were filtering water through the gills, i.e., feeding. Only the shell movements of the oysters were recorded on the kymograph, while no data were obtained on the quantities of water pumped by the oysters during the different tidal stages. Obviously, no definite conclusions could be formed concerning the latter subject because of the almost complete lack of experimental evidence regarding this matter.

Nelson's method of interpreting the data should also be mentioned. In analyzing his material on shell movement of the oysters Nelson (1921) takes into consideration only the numbers of openings and closures of the shells during the different tidal periods. This method has already been criticized by Galtsoff (1928) who stated that "The examination of the number of closures and openings occurring during a given period of time does not convey a true idea of the activity or inactivity of the oyster. A better understanding can be gained by counting the number of hours the oyster was closed or open during a given period of a day." Obviously, Galtsoff's suggestion is well founded.

Our observations and experiments supplied the evidence that the oysters of Long Island Sound and Milford Harbor fed actively during the flood and ebb periods, and that during the ebb their feeding was often more energetic than during the flood. Observations of this nature could probably be made in many other bodies of water where changes in the tides are not accompanied by pronounced ecological changes. This conclusion appears to be logical because it is quite improbable that, if other conditions of the environment remain favorable, a change in the direction of the tidal current alone would affect the oysters. It is, to a certain extent, supported by our experiments in which pairs of oysters, employed in our studies of the shell movements, were always placed so that the hinges of their shells pointed in opposite directions. Thus, while the gills of one oyster faced the flood, the gills of the other animal were turned away from the direction of the current. Yet, no difference suggesting that an oyster in a certain position was more active on the flood or ebb was generally noted.

No correlation between the rate of water pumping of the oysters and the time of day was demonstrated by our experiments. Neither was it found that the duration of the opening and closure of the shells was affected by periods of light or darkness. In nature many oyster beds are located at a considerable depth. Very often the water flowing over the beds contains large quantities of suspended matter which stop the penetration of a large quantity of light before it reaches the bottom on which the oysters live. These oysters, therefore, normally exist in near-darkness even during very strong daylight. It is very doubtful that if other conditions remain favorable, the slight change in the intensity of illumination caused by the approach of night could have such a pronounced effect on the oysters that they would either begin to feed at a much slower rate or stop feeding entirely.

If the rate of feeding of oysters were markedly decreased during the nights and during the ebb periods, the existence of these mollusks would be under a rather unfavorable condition. Because the periods of darkness are often followed by ebb, there would be times when the feeding activities of the oysters would be continuously depressed for a period of approximately 18 hours. This condition would occur in September and October when the nights become long. However, it is a

well known fact that during these two months the oysters of our waters undergo very rapid improvement in condition storing large quantities of glycogen in their bodies. Naturally, such an improvement could not be possible if, during this time of the year, the oysters had to exist under the conditions compelling them to be relatively inactive during approximately 12 hours of darkness and also during the 6 hours of ebb, a total of 18 hours per day.

As may be seen from this discussion, our conclusions regarding the activities of oysters during ebb and during periods of darkness do not agree with those of Nelson and Orton (ref. cit.). However, as Dr. Nelson suggests in recent personal communication with the senior author, the cause of the divergence may be attributed to the marked reduction of the pH during ebb and at night in the waters where Nelson's experiments were conducted. During the outgoing tide those areas received large quantities of swamp water which noticeably lowered the pH. Also, according to Nelson "These waters are but slightly buffered; hence at night with the respiration of algae and of animals and decomposition the water may become acid by morning." Such changes are indicated in one of Nelson's reports (1924). The conditions, however, are different in other basins, such as in many sections of Chesapeake Bay (Loosanoff, 1932) and Long Island Sound (Loosanoff and Engle, 1940) where the pH does not closely approach the neutral point.

In general, our experiments have shown that under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor and Long Island Sound. Although the differences in the behavior between the individual oysters are of considerable magnitude, these mollusks, nevertheless, appeared to be feeding all or most of the time their shells remained open which, with a temperature range from 17.0 to 28.0° C., was approximately 94 per cent of the total time.

In presenting the final conclusions it should be once more emphasized that we do not interpret our results as applicable to all oyster growing areas of this coast. While our observations hold true for the areas where the experiments were conducted, and also, probably, for the waters where the ecological conditions resemble ours, it is realized that in other basins, where during ebb the oysters are exposed to unfavorable environment, different conditions may prevail. Nevertheless, the material presented in this article clearly indicates that the conclusions of Nelson (ref. cit.), which, no doubt, are representative for Huey's Creek, should not have been generalized and presented as applicable to the American oyster as a species (Orton, 1929).

SUMMARY

1. Examination of approximately 1400 oysters collected during the different tidal stages in Long Island Sound and Milford Harbor failed to show any definite period when the stomachs of these mollusks displayed absence of food.

2. During all hours of the flood and ebb, including the low water period, the predominating majority of the oysters contained large quantities of food, whereas individuals with empty stomachs were found only occasionally.

3. The relative quantities of food found in the oyster stomachs during the ebb period were at least equal to or sometimes even exceeded those recorded during the flood.

4. Analysis of the kymograph records of the rate of water pumping by the oysters showed that they fed very actively at all stages of the tide, and that the rate of feeding during ebb was at least equal to or sometimes even more rapid than during the flood stage.

5. During the ebb some of the oysters pumped on an average of 25,000 to 27,000 cc. of water per hour, while the maximum rate of pumping in some instances ranged from 31,000 to 34,000 cc. per hour.

6. The efficiency of the feeding mechanism of an oyster may be well attested by the fact that the volume of water passed during one hour through the oyster gills may be more than 1500 times greater than the volume of the oyster's body.

7. Within the temperature range of 17.0 to 28.0° C. the oysters remained open on an average of 22 hours and 39 minutes, or 94.3 per cent, and were closed 1 hour and 21 minutes, or 5.7 per cent of a 24-hour period.

8. During the periods of flood the shells of the oysters remained open on an average of 93.4 per cent of the time, whereas during the ebb periods the shells were open 95.2 per cent.

9. During the periods of darkness the percentage of oysters with full stomachs was comparable to that of the individuals examined during the day time.

10. During darkness the oysters were found feeding very actively. The average rate of pumping at night was not lower than during the daytime.

11. The shells of the oysters remained open 94.4 per cent of the total time during daylight, and 93.8 per cent during the period of darkness. No correlation was found between the periods of closure of the shells and darkness.

12. Under favorable conditions neither tidal changes nor changes in the time of day affect the rate of feeding of oysters of Milford Harbor. These mollusks were found to be feeding all or most of the time when their shells remained open.

13. The results of this investigation do not lend any support to the generally accepted theory that the American oyster does not feed late at night and in the early morning, and is relatively inactive on the ebb tide.

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AUTOSOMAL ELIMINATION AND PREFERENTIAL SEGREGATION IN THE HARLEQUIN LOBE OF CERTAIN DISCOCEPHALINI (HEMIPTERA)

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INTRODUCTION

It has been known for some time that the various lobes in the testis of many species of pentatomid Hemiptera show constant differences in the size of their cells. Bowen (1922a and b) who investigated this condition most recently, concluded that it is attributable mainly to differences in the volume of cytoplasmic elements. That is a finding which I can only confirm; if differences in the volume of chromatin exist they must be very small. However in some species there is a testicular lobe whose cells differ from those of other lobes not only in size but which has also evolved an entirely novel process of maturation. The main features of this maturation are in several instances almost fantastic in character, and the evolution and constant occurrence of such "harlequin" lobes is a matter of some interest.

It should be emphasized that we are dealing here not with accidental or sporadic occurrences. In the species concerned the harlequin lobe is found in each testis of every male. Moreover for any given species it is always a certain and very definite lobe that is thus characterized (in the Discocephalini here treated it is the fifth) and hence it is clear that its development involves conditions that are fundamentally and firmly established in the species as it is now constituted.

Harlequin lobes have so far been encountered in three species of *Loxa*, a species of *Mayrinia* (Schrader, 1945a and b), and in a species of *Brachystethus* (Schrader, 1946). In the last named the departure from a normal meiosis lies primarily in the autosomes which are shunted out of the spindle in both divisions; in *Loxa* and *Mayrinia* the aberrancy takes the form of amitosis and fusion in the spermatocytes, resulting in a highly variable heteroploidy. The meiotic anomalies of *Brachystethus* and *Loxa* thus appear to be in no way related and yet it seems only natural to assume as a working hypothesis that the evolution of harlequin lobes involves similar basic conditions in all the species involved.

It is likely that further investigations will discover that harlequin lobes are present in a great many species. To the five species mentioned above and the three taken up in the present paper may be added at least three further species which I have not as yet fully analyzed—a total of eleven. Taxonomically speaking, these species cover a wide range. *Loxa* and *Mayrinia* represent typical genera of the tribe of Pentatomini; *Brachystethus* is so closely related to the Edessini as to furnish almost a "bridging" genus between that tribe and the Pentatomini; and the Discocephalini constitute a tribe so distinct from the other pentatomid tribes that it has sometimes been elevated to the rank of a subfamily (Lethierry and Severin, 1896).

Conditions in the females of all these species are still unknown, except as they were used in all instances to check the identification of the sex chromosomes in the males. Cytologists need hardly be told that this gap in our knowledge is due mainly to the technical difficulties that render a study of meiosis in the egg so onerous a task.

I should like to point out again as I have done in my study of *Brachystethus*, that the investigation of the harlequin lobe is made under the almost ideal conditions of a natural experiment. The adjoining lobes of the same testis are perfectly normal and serve continually as a control; frequently the normal and aberrant cells can be studied in one and the same field of the microscope.

MATERIAL AND METHODS

The Discocephalini investigated are: *Mecistorhinus melanoleucus* Westwood (one male from Panama); *Mecistorhinus tripterus* Fabricius (four males and two females from Costa Rica); *Mecistorhinus sepulcralis* Fabricius (one testis each from eight different males and the ovaries from one female, all from Piracicaba, Brazil); *Neodine macraspis* Perty (five males from Costa Rica); and *Platycarenum notulatus* Stål (three males and one female from Costa Rica). The last named species has no harlequin lobe and is only briefly mentioned in the following pages.

My thanks are due to the eminent hemipterist, Mr. H. G. Barber, who identified all the species of *Mecistorhinus*. To Professor S. de Toledo Piza of the University of São Paulo, Brazil, I am deeply indebted for the material of *Mecistorhinus sepulcralis*.

Fixation was made in either Bauer's convenient modification of Allen's Bouin or in Sanfelice. As in all my recent studies of mitosis, I have employed three staining methods. The Feulgen technique is indispensable as a test for chromatin; gentian violet (in Smith's modification of Newton's method) is often very useful for a study of the detailed structure of the chromosomes but even when combined with erythrocin is not an efficient stain for the spindle apparatus in pentatomids; whereas Heidenhain's hematoxylin remains beyond all comparison the best means for bringing out asters, centrioles and spindle fibers. As noted above, female material was studied only to check identification of the sex chromosomes in the male.

In all the Discocephalini where a harlequin lobe occurs, it is the fifth of seven lobes in each testis. In every case it is two or three times as voluminous as any other lobe although its spermatocyte cells after the leptotene stage are smaller than those of the rest of the testis. The fourth and sixth lobes which flank it on either side carry exceptionally large but otherwise normal cells, whereas the remainder conform to more orthodox proportions.

In the following pages the different species are taken up separately, the detailed analysis of *Mecistorhinus melanoleucus* being followed by briefer comparative accounts of the other forms. As far as possible, the interpretative treatment is relegated to the discussion that terminates the paper.

MECISTORHINUS MELANOLEUCUS

Normal lobes

Except for certain features which are pertinent to an analysis of the peculiarities of the harlequin lobe, no detailed account of the spermatogenesis in the six normal

lobes need be given. It conforms closely in its general course to that which has often been described in other pentatomids.

The diploid set of fourteen chromosomes is marked by one exceptionally large pair of autosomes. This pair stands out almost as conspicuously as does the X chromosome of *Protenor*. Here however, the sex chromosomes are relatively small, the Y being the smallest member of the complement and the X little if any larger than the smallest of the autosomes (Fig. 2).

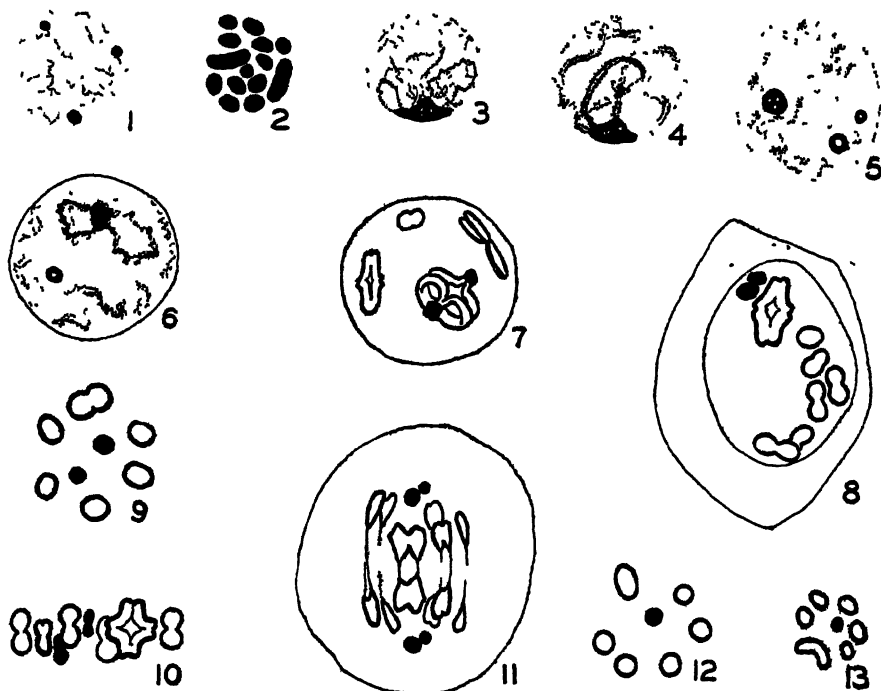
One of the exceptional features lies in the heteropycnosis that marks not only the sex chromosomes but also certain of the autosomes. Already in the early generations of the spermatogonia there are from three to five heteropycnotic bodies that stand out prominently in the resting phase (Fig. 1). There is no prochromosome stage intervening between the last spermatogonia and the leptotene stage of meiosis. In the leptotene and synaptic stages the heteropycnotic bodies are usually aggregated in a single mass on the nuclear periphery and it is at this locus also that the fine leptotene threads come together in a bouquet formation (Fig. 3). But in some cells there are two heteropycnotic bodies during these stages, the second and smaller one usually lying at some distance from the first and not necessarily at the periphery. The advent of the pachytene and diplotene stages sees little change (Fig. 4) in these conditions of heteropycnosis and it is only when they in turn give way to the confused stage that the single heteropycnotic aggregate is dissociated again. In this peculiar phase when staining conditions and despiralization temporarily convert most of the chromosomes into pale and flocculent threads there may again be three, four, or five heteropycnotic bodies (Fig. 5). This variation in number would seem to indicate that the mutual and nonspecific attraction that brings heteropycnotic chromosomal bodies together at certain stages is not very strong and it is likely that accidents of position determine these numbers to some extent.

In early diakinesis, as the chromosome threads again become definite in outline, the topographic relationships are once more open to analysis. Now the great majority of cells show only two heteropycnotic bodies, one of which becomes less and less conspicuous as the threads shorten and condense¹ while the larger one is seen to be intimately associated with the big bivalent (Fig. 6). Somewhat later, when the paired chromosomes have assumed the typical cross and ring formations of late diakinesis this large heteropycnotic body has disappeared, but there are then two smaller bodies, one associated with each of the two spreading arms of the large bivalent (Fig. 7). There is no doubt about the identity of these bodies. The larger is the X and the smaller the Y, the two together constituting the larger heteropycnotic body or chromosome nucleolus of earlier diakinesis. The dissociation of this single nucleolus into its two components is perhaps due not only to the strains that attend the separation of the arms of the large bivalent but may be a part of the regular cycle that in other species also sees the reappearance of the separate sex chromosomes at this stage. What is more remarkable is their persistent union with the arms of the autosomal bivalent, a union which is not broken until shortly before metaphase.

The rather even peripheral distribution of the diakinetid bivalents disappears

¹ The present state of our knowledge concerning the changes in the chromosome during a complete mitotic cycle is still unsatisfactory. Almost certainly both coiling and nucleination are involved, but the relative importance of these two factors remains undetermined. For that reason the terms "condensation" and "diffuseness" are here used in a purely descriptive sense.

just before the disintegration of the nuclear membrane. They then lie helter skelter in the nucleus and may even come into contact with each other. It is at this time when the chromosomes are in the final stages of condensation that the two sex chromosomes sever their connections with the large tetrad, though their former as-



Mecistothorus melanoleucus—Normal Lobe

- FIGURE 1. Early prophase in spermatogonial cell; three chromatin nucleoli (Feulgen).
 FIGURE 2. Spermatogonial metaphase; Y is smallest of the 14 chromosomes (Feulgen).
 FIGURE 3. Leptotene stage (Feulgen).
 FIGURE 4. Diplotene stage (Feulgen).
 FIGURE 5. Confused stage (Feulgen).
 FIGURE 6. Early diakinesis; XY nucleolus attached to large tetrad (Feulgen).
 FIGURE 7. Late diakinesis; X and Y attached to separate arms of large tetrad (Hematoxylin).
 FIGURE 8. Prometaphase; X and Y still close to large tetrad (Hematoxylin).
 FIGURE 9. Metaphase I; polar view (Hematoxylin).
 FIGURE 10. Metaphase I; side view (Feulgen).
 FIGURE 11. Anaphase I; large tetrad lagging (Gentian violet).
 FIGURE 12. Metaphase II; polar view (Hematoxylin).
 FIGURE 13. Telophase II; 6 autosomes + Y (Feulgen).

sociation is frequently indicated by their close proximity to it (Fig. 8). It is this stage also that is marked by an elongation of the nucleus as a whole in the polar axis, a change that plainly involves interaction with the two centers located at the periphery of the cell.

About the meiotic divisions themselves, little need be said. Metaphase I is quite typical in its conformation, with the now separated X and Y usually in the center of a ring of six tetrads (Fig. 9). It is however worthy of note that the large bivalent is somewhat slower than the rest of the autosomes in its condensation and side views of the first equatorial plate still show it as a cross tetrad (Fig. 10). At anaphase the X and Y divide equationally and arrive at the poles before the rest of the chromosomes, whereas the large bivalent (often showing the tertiary split) lags in its division and is distinctly slower than the other autosomes in its anaphasic progress (Fig. 11).

The second division also witnesses some lagging on part of the large autosome, but this is not as striking as in the first division. The touch and go pairing of the X and Y occurs as usual, and in metaphase they line up in the spindle axis so that in polar views one is superimposed on the other (Fig. 12). They then separate to opposite poles and the spermatids receive the typical pentatomid complements of $6A + X$ and $6A + Y$ respectively (Fig. 13). The departures from the orthodox process of meiosis thus do not affect the results, which conform to the regular pentatomid scheme.

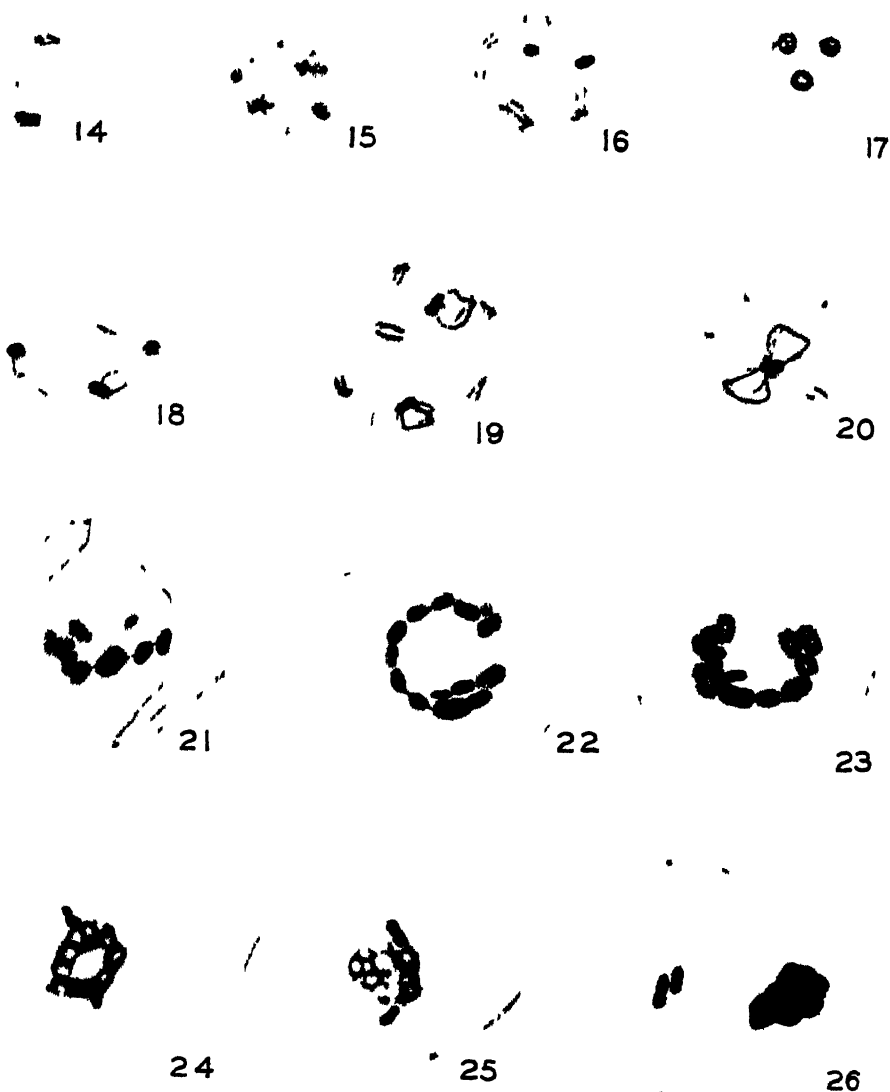
Harlequin lobe

Spermatogonia and meiotic prophases

The spermatogonial stages in the harlequin lobe differ in no discernible way from those of the normal lobes. Here too there are, in Feulgen preparations, from three to five heteropycnotic bodies in the resting phase and the succeeding stages closely parallel the normal course of events. As in the normal lobes there is no prochromosome stage. The meiotic leptotene duplicates that of the other lobes in the number and disposition of heteropycnotic bodies, but the chromosome threads do not seem to be as finely drawn out and delicate as they normally are. This difference however is too slight to furnish a secure basis for contrast (Fig. 14). Succeeding this stage the developments follow a path that diverges widely from the usual one.

There is neither a synapsis nor a pachytene stage. During the period in which these developments occur in the normal lobes, the chromosome threads of the harlequin lobe merely abandon their bouquet orientation and undergo a progressive condensation. As a result the nucleus then shows twelve somewhat loosely coiled autosomes and the two more condensed sex chromosomes, clear evidence that any sort of pairing that may have occurred unobserved prior to this time has now been abrogated (Fig. 15). The picture presented is a surprisingly close approximation of the prochromosome stage as it occurs normally in some Hemiptera, and in this respect has some resemblance to the conditions in the harlequin lobe of *Loxa* (Schrader, 1945b) where such a post-leptotene condensation is also encountered. But in *Loxa* there is a true prochromosome stage as well which occurs quite normally prior to the evolution of the leptotene threads. Both there and in *Mecistorrhinus* no confusion is possible for not only does the true prochromosome stage occur much higher in the testis, but its nuclei are considerably smaller than are the ones here in question.

This post-leptotene condensation culminates in shortened, fuzzy chromosomes that show an equational split (Fig. 16), undoubtedly a condition corresponding to



Mccistorhinus melanoleucus—Harlequin Lobe

FIGURE 14. Leptotene stage (Gentian violet).

FIGURE 15. Post leptotene condensation; X and Y heteropycnotic (Feulgen).

FIGURE 16. Diplotene stage in univalents (Gentian violet).

FIGURE 17. Late confused stage (Feulgen).

FIGURE 18. Early diakinesis; sex chromosomes attached to separate large autosome (Gentian violet).

FIGURE 19. Mid-diakinesis; 12 univalents autosomes, with each large autosome combined with one sex chromosome (Gentian violet).

the diplotene stage of the normal lobes. Throughout this period two pairs of chromosomes are readily recognizable; they are the two large autosomes and the two heteropycnotic sex chromosomes. Each large autosome has its ends united so as to form a split ring, a configuration that very probably arises from the mutual attraction of its heteropycnotic terminal regions. The two heteropycnotic sex chromosomes show no such attraction at this time and usually lie well separated, evidence that heteropycnotic attraction is confined to certain conditions of the heterochromatin.

The confused stage which now intervenes, temporarily halts a further close analysis of progressive chromosome changes. The autosomes once more become diffuse and uncoiled and at the height of the stage stain very lightly. Usually three heteropycnotic bodies are present at this time, but there may be as many as five (Fig. 17). In the latter case the bodies are smaller, generally speaking, which would indicate that the variations in number are due to some vagaries in mutual attraction and aggregation. The two sex chromosomes and the ends of the two autosomes would account for six such bodies which suggests that some aggregation is nearly always present.

With the termination of the confused stage and the beginning of diakinesis, the individual chromosomes once more appear as such. There are then three heteropycnotic bodies and two of these are seen to be associated with the two large autosomes (Fig. 18). The third shows no such definite association and gradually disappears. In mid-diakinesis a more exact analysis of these conditions becomes possible. At this time there is a total of either eleven or twelve chromosomal bodies in every nucleus. When there are twelve, the two autosomes are quite independent of each other and may lie far apart. Each of them has a large chromatin nucleolus or heteropycnotic body attached to it at the place where the ends are still joined in ring formation (Fig. 19). When on the other hand there are only eleven bodies, these two chromatin nucleoli have come together, and through them the two large, ring formed autosomes have joined in a figure eight (Fig. 20). The two chromatin nucleoli represent the X and Y chromosomes and again, the most natural explanation of such configurations would seem to lie in the forces of heteropycnotic attraction; the heteropycnotic ends of the large autosomes are drawn together to form rings, and the heteropycnotic sex chromosomes later become attached to these regions and to each other for the same reason. It is rather strange that no case has been encountered in which both sex chromosomes have become joined to only one of the large autosomes, since nonspecific heteropycnotic attraction might be expected to give rise occasionally to such configurations. However, nuclei of this stage in which the chromosomes are open to a clear analysis are not common and

FIGURE 20. Mid-diakinesis; both sex chromosomes and both large autosomes in one combination (Gentian violet).

FIGURE 21. Equatorial ring side view (Hematoxylin).

FIGURE 22. Equatorial ring slightly later; polar view (Gentian violet).

FIGURE 23. Autosomes in precocious return to diffuse condition; X and Y still heteropycnotic (Gentian violet).

FIGURE 24. Formation of autosomal aggregate; X and Y heteropycnotic (Gentian violet).

FIGURE 25. Dissociation of X and Y from autosomal aggregate (Gentian violet).

FIGURE 26. Metaphase I; X and Y on middle spindle and autosomal aggregate displaced (Hematoxylin).

the fourteen examples which have been studied hardly constitute a sufficiently large number to justify the conclusion that they do not occur.

Prometaphase

Shortly before the breakdown of the nuclear membrane a significant reorientation of the chromosomes takes place. This is at about the time that the nucleus elongates toward the peripherally located centers. The chromosomes, still not fully condensed, then are shifted to the middle region between the centers and since they remain in close proximity to the nuclear wall and have lost the property of mutual repulsion, they tend to form a more or less circular row or chain in the equator. Some of the components of such chains may be in actual contact with each other, while others may be connected by Feulgen positive bridges or show no attachment at all (Figs. 21, 22, and 72). It is likely that such bridges are similar in nature to those seen later at metaphase (see for instance Ris, 1942), but whether they represent viscous connections that persist after a former contact or are indicative of a "reaching out" of chromosomes toward each other, it is impossible to decide.

When the nuclear membrane finally disappears, this picture undergoes marked and sudden changes. The chain of chromosomes, now free of the influence of the membrane, seems to collapse inwardly, frequently forming a closed ring at first and then an irregular aggregate in the middle of the nuclear space. In the many cells seen at this and the following stages no instance of more than a single aggregate has ever been observed, a point of difference with the case of *Brachystethus* (Schrader, 1946). Concurrently with these changes of orientation there occur alterations in the chromosomal structure. These are marked especially by a partial return to the diffuse condition in the autosomes, with an accentuation of the equational split. The two large autosomes do not seem to become quite as diffuse as the rest, but this difference is not a striking one at best. This return to a more diffuse state causes the autosomal aggregate to appear as a spongy and vacuolated mass in both gentian violet and Feulgen preparations and this condition is maintained for the major part of the first division (Fig. 23). Hematoxylin slides allow no such structural diagnosis for there the aggregate is nearly always homogeneously and intensely stained.

The behavior of the sex chromosomes is remarkable during the prometaphase and the establishment of the metaphase itself. At the time of the equatorial ring formation, just prior to the disintegration of the nuclear membrane, they are still very close or even in contact with the large autosomes. Almost always they lie on the inner side of the ring and not in seriation with the rest of the chromosomes (Fig. 22). They seem to be almost fully condensed at this time and are recognizable in most cells. When the autosomal chain collapses to form the irregular aggregate, this distinction becomes even more marked, for in contrast to the autosomes they then maintain their condensed state and in addition tend to protrude from the spongy mass of autosomes (Fig. 24). This protrusion seems to be an indication of interaction with the two centers, for the sex chromosomes not only make their appearance on the side toward one of the poles but begin to place their long axis in alignment with the polar axis (Fig. 25) which is a placement assumed also at the ensuing metaphase.

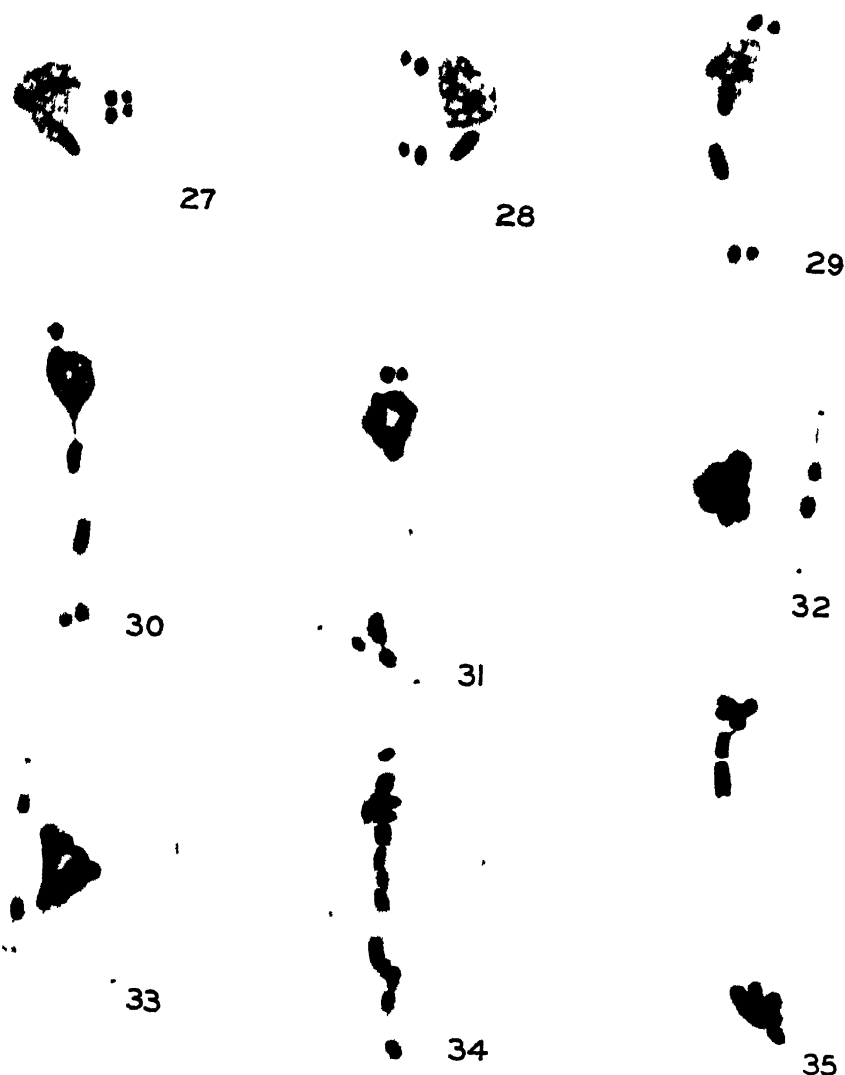
The first division

The clumping of the partially diffused autosomes brings about a rather anomalous situation and the establishment of the metaphase can be followed only through the behavior of the sex chromosomes. These appear to be quite normal in their further maneuvers. They finally become completely detached from the spongy aggregate of autosomes and take up an equatorial position side by side. During this movement several other developments occur simultaneously. Chromosomal fibers appear connecting the sex chromosomes as well as the aggregate with the poles, and at the same time the whole mass of autosomes is shunted out of the middle region toward the side of the cell. This shift must be rather sudden, for intermediate stages are very rare. In extreme instances the displacement may bring the autosomal aggregate very close to the side of the cell, though never touching it, and in every case it comes to lie farther from the polar axis than from the cell wall. While in this position, two points are to be noted: the autosomal aggregate remains connected with the poles through definite chromosomal fibers, and even in its displacement it maintains an equal distance from both poles (Figs. 26, 73, and 74). The whole reaction is obviously closely akin to a similar one observed in the pentatomid *Brachystethus* (Schrader, 1946).

The two sex chromosomes apparently are not affected by the anomalous behavior of the autosomes. They lie side by side in a compact and narrow spindle of normal length and undergo an orthodox equational division. In some cells the chromatids of the Y separate faster than those of the X and may precede them to the poles. As soon as the anaphase movement of the sex chromosomes is initiated, the autosomal aggregate once more approaches the polar axis, and by mid-anaphase is usually close to or even in contact with the sex chromosome spindle. This return also occurs in the equatorial plane of the cell, and is correlated with a shortening of the chromosomal fibers as well as the lengthening of the interpolar distance and cell as a whole—both of which will of course bring the autosomal aggregate closer to the polar axis again (Figs. 27 to 29).

Although the autosomes, aggregated as they are, pass through these maneuvers as a unit, there is evidence from the beginning that one of them plays a special role. Already at the first trace of division in the X and Y chromosomes, a single large chromosome protrudes from the autosomal clump, showing a well formed chromosomal fiber connection with one center and clearly oriented toward it. In such a position it appears more condensed than the rest of the autosomes, a condition which would be difficult to discern while it is still in the midst of the vacuolated, unevenly staining aggregate (Figs. 27 and 28). The reaction of this autosome to the pole is quite independent of the sex chromosomes, but it is obviously hindered in its movements—probably because of the "stickiness" that tends to hold all the autosomes together. As a result the two sex chromosomes are well on their way toward the poles before this autosome has disengaged itself from the encumbrance (Figs. 29 and 75).

Soon after it has left the aggregate, a second large autosome begins to dissociate itself from the rest of the autosomes. The extent to which it succeeds in this is highly variable in different cells, but in most cases it at least protrudes from the mass before the division is finished (Fig. 30). Often, while the first autosome is still fairly



Mecistorhinus melanoleucus—Harlequin Lobe

- FIGURE 27. Early anaphase I; autosomal aggregate beginning to return (Gentian violet).
 FIGURE 28. Mid-anaphase I; (Gentian violet).
 FIGURE 29. Late anaphase I (Gentian violet).
 FIGURE 30. Early telophase I (Gentian violet).
 FIGURE 31. Late telophase I (Hematoxylin).
 FIGURE 32. Early anaphase II—large cell (Gentian violet).
 FIGURE 33. Mid-anaphase II—large cell (Hematoxylin).
 FIGURE 34. Early telophase II—large cell (Hematoxylin).
 FIGURE 35. Telophase II—large cell (Hematoxylin).

close to the aggregate, it shows a Feulgen-positive connecting thread with the second one, although this is usually severed very shortly (Fig. 29).

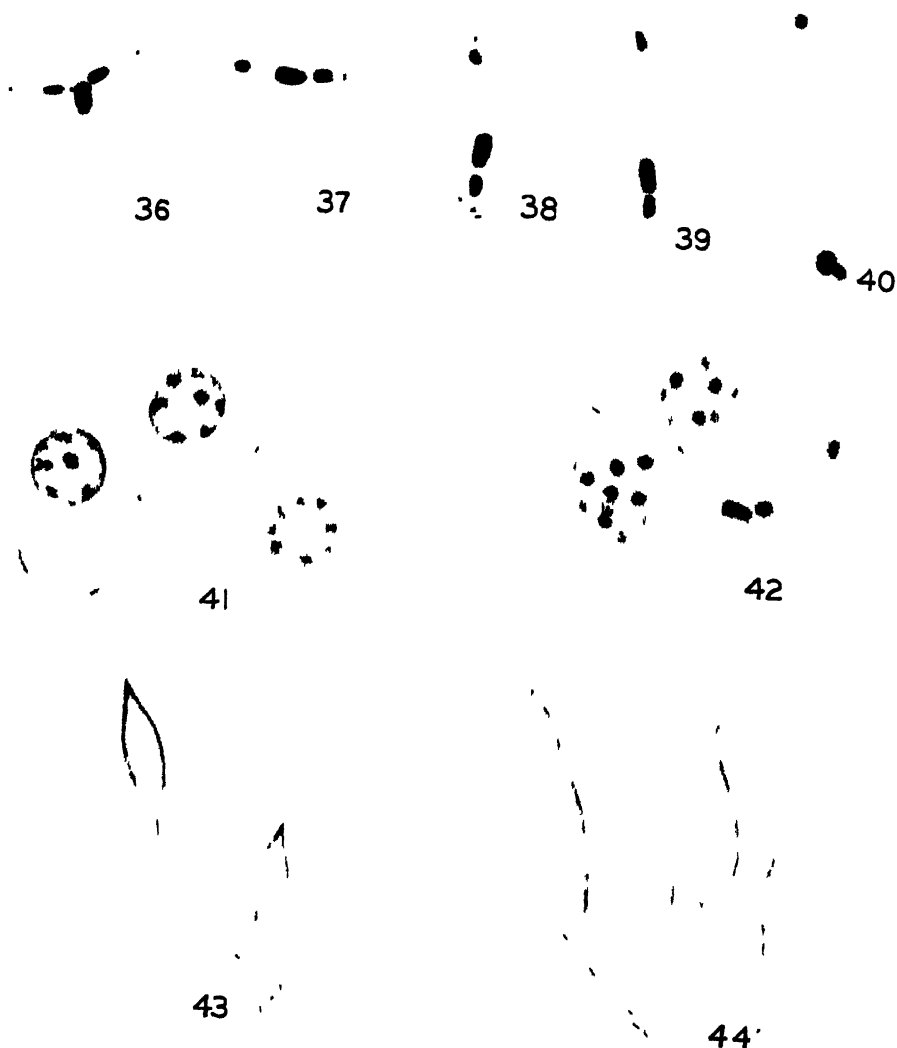
The rest of the autosomal clump, which is by now very close to the polar axis, also shows some response to the mitotic forces and frequently undergoes some elongation in the polar direction. Its effective movement however is always opposite in direction to that of the first autosome and hence it approaches the other pole. The second large autosome may sometimes almost reach the middle of the cell, but in only a single case has it proceeded so far that the ensuing cleavage constriction promises to include it in the same cell with the first autosome. Indeed, a count of 100 small second spermatocytes (those which do not receive the main part of the autosomal aggregate) has revealed no such accidental inclusion and it must be very rare. Further, in all observed cases (of which there are many dozens) this second autosome has rejoined the aggregate by the time that the second division is begun so that its mitotic motion must finally be reversed (Fig. 31). Possibly it initially follows the large autosome only because it is dragged along by the connecting thread. The final result of these maneuvers is that the large autosome and the rest of the autosomal aggregate always go to opposite poles. Since the former is the first to evince any reaction to the mitotic forces, one is almost forced to the hypothesis that it determines the direction of movement on the part of all the remaining autosomes.

These two autosomes that tend to disengage themselves from the aggregate are patently larger than any but the two largest chromosomes of the diploid set. At the same time they do not seem quite to reach the size of that large pair and it may therefore be that we are dealing with the two chromatids of only one of the latter. But admittedly the changes in the state of autosomal condensation during meiosis make it difficult to decide the matter on the basis of size alone. The later behavior of these exceptional chromosomes in the spermatids would however seem to support their identification as chromatids rather than whole chromosomes.

The second spermatocytes resulting from this first division, anomalous though it may be, are thus very constant in composition. Half of them contain the two sex chromosomes and only one autosome; the rest carry all the remaining autosomes as well as the sex chromosomes. The latter are much the larger cells of the two, as might be expected (Fig. 31).

The second division

Large cell: The chromosomes carried by these second spermatocytes comprise the sex chromosomes as well as all of the autosomes except the single one in the smaller cell. There is no interkinesis. In its general aspects the division of this cell simulates the first division. As the X and Y take their position on the new spindle, chromosomal fibers are also formed between the two poles and the autosomal aggregate. Almost simultaneously, the latter is shifted toward the side of the cell, the displacement being very similar to that observed in the first division (Figs. 32 and 76). However the aggregate now begins to be less spongy in appearance and there are other indications that its autosomal constituents are once more undergoing condensation (Fig. 33). During the anaphasic separation of the X and Y, the aggregate is again drawn toward the middle of the cell. It may be noted that when it reaches the compact little sex chromosome spindle, its surface of contact with it becomes smooth and concave, indicating that it is under some pressure (Fig. 33).



Mecistorhinus melanoleucus—Harlequin Lobe (except Figs. 41 and 43)

FIGURE 36. Beginning of Division II—small cell; large autosome connected with both X and Y (Hematoxylin).

FIGURE 37. Early anaphase II—small cell (Hematoxylin).

FIGURE 38. Late anaphase II—small cell (Hematoxylin).

FIGURE 39. Early telophase II—small cell (Hematoxylin).

FIGURE 40. Late telophase II—(Hematoxylin).

FIGURE 41. Early spermatids—normal lobe (Hematoxylin).

FIGURE 42. Early spermatids—harlequin lobe (Hematoxylin).

FIGURE 43. Late spermatids—normal lobe (Hematoxylin).

FIGURE 44. Late spermatids—harlequin lobe (Hematoxylin).

Then as the anaphase progresses and the cell and spindle elongate, the aggregate is drawn out between the two poles and many of its component autosomes are thus strung out in a roughly linear order (Figs. 34 and 78). But the tendency to stick and adhere to each other remains strong so that even when several individual chromosomes have been pulled out of the aggregate they usually still show thick, Feulgen-positive connections with each other and with the remaining aggregate (Figs. 34 and 35). The division so far as the autosomes are concerned is therefore obviously a haphazard one and the aggregate is frequently distributed very unevenly to the two spermatid cells.

The spermatids resulting from the division of the large second spermatocyte are therefore exceedingly variable in composition. Since the sex chromosomes segregate normally, all spermatids carry either an X or a Y, but the number of autosomes is largely a matter of chance. Nevertheless no cases have been observed in which the latter have all gone to one pole.

Small cell: As is the case with the large second spermatocytes there is no interkinesis of any kind and the final stages of the first division merge directly into the beginnings of the second. Indeed before the anaphase movement of the first division has been completed, the two daughter centrioles at each pole (each centriole appears double already at metaphase) have separated and begin their migration to establish the polar axis of the second division, at right angles to the first (Fig. 31). In some cells the sex chromosomes respond and orient to these new poles before they have completed the anaphasic movement. But apparently this precocious movement is then reversed, for in slightly later phases when the new axis has been established, the two sex chromosomes are near each other or in actual contact in the middle of the cell. Here too now lies the large autosome which has been delayed in its arrival at the pole. It may be in contact with neither, either or both sex chromosomes, or it may be connected with either or both through Feulgen-positive bridges (Fig. 36).

Chromosomal fibers are formed already before the three chromosomes have gathered at the midpoint, but so far as can be seen the tiny spindle is concerned only with the X and Y. No chromosomal fibers can with certainty be traced to the autosome. The sex chromosomes behave just as they do in a normal cell and after meeting in the middle of the spindle they separate to opposite poles in a regular segregation.

It is the behavior of the autosome that presents some puzzling aspects, as it already has done in the first division. The outstanding feature of this behavior lies in the fact that it nearly always goes to the same pole with the X. In so doing it appears to have little or no independent mitotic movement, acting merely as a satellite of the sex chromosome. Its dependence on the latter is shown not only by the absence or poor development of chromosomal fibers already mentioned, but is indicated also by its behavior before and during anaphase. In the grouping prior to the division, the autosome sometimes lies between the X and the adjacent pole, but when the anaphase movement is under way it always trails the sex chromosome (Figs. 36 to 40, and 77). Since during most late anaphases the autosome is attached more or less closely to the X, its behavior might be attributed to a simple adhesion between the two were it not for the fact that many earlier anaphases show no such connection. The latter thus frequently seem to be established after the mitotic movement is under way. Indeed, connections of this sort cannot be decisive in any case since prior to the division the autosome often lies in contact with both

sex chromosome or shows a Feulgen-positive bridge to the Y and not to the X (Fig. 36). Evidently this is later broken so that the mitotic association with the X must involve some selective action not dependent on such physical bonds. Whatever the underlying mechanism may be, the results of the division admit of no doubts concerning the constancy of the relationship between these two chromosomes. In 100 clear side views of late anaphases there were only six that admitted the possibility of an association of the autosome with the Y. In two of these the autosome is clearly much closer to the Y than the X and probably going to the same pole, whereas in the remainder the identification of the X and Y is not certain. In close to 95 per cent of the cases therefore, the autosome accompanies the X to the pole and not the Y. Generally speaking then, the small spermatids which come from this division are of two types: one, which carries the X and the autosome; and another which contains only the tiny Y.

Spermatids and sperms

The two anomalous spermatocyte divisions of the harlequin lobe thus give rise to four main types of spermatids: X + one large autosome; Y; X + a variable number of autosomes; Y + a variable number of autosomes. Since in the spermatid the autosomes scatter again and tend to be distributed peripherally on the new nuclear membrane before becoming diffuse, rather dependable counts are often possible. Both of the small types of spermatids are readily recognizable and it is to be noted that the close association between the X and the autosome of the preceding telophase is maintained into an advanced spermatid stage (Fig. 42). In the large spermatids the number of chromosomes may be as low as five or six and frequently higher than twenty. The latter counts constitute conclusive evidence that the chromatids of the univalent autosomes have separated from each other, since the full, normal haploid number of the species is only seven (compare the normal spermatids of Fig. 41 with those of the harlequin lobe in Fig. 42). Since the autosome that is associated with an X in one of the small spermatids undergoes no such separation into smaller units, there is strong presumptive evidence (to support that which was adduced earlier) that it represents a chromatid which has already separated from its sister chromatid—the second large autosome of the first division.

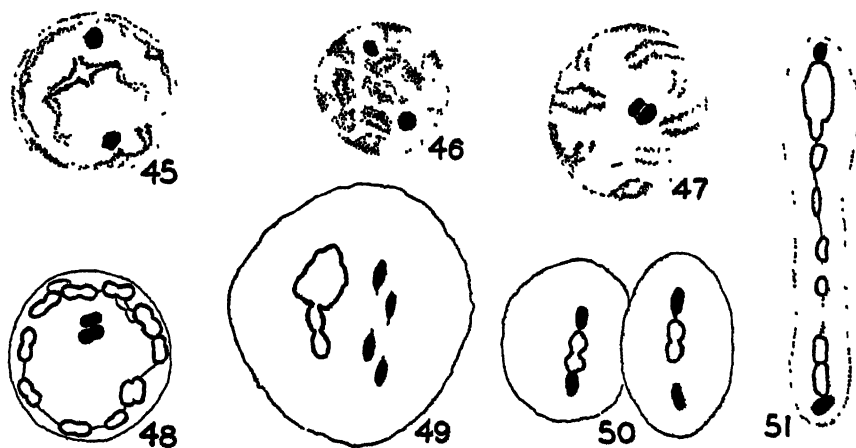
Formation of sperms seems to proceed normally in all cells until the stage when the elongation nucleus shows the pointed apex which indicates the presence of the acrosome (compare Fig. 43 with Fig. 44). At about this time, when the chromosomes have become very diffuse and the contents of the sperm head seem structureless, the small types of sperms gradually stain more and more faintly. It finally becomes impossible to recognize them, whereas the larger types assume the attenuated, intensely stained form of the head that is also encountered in the normal lobes. It is more than probable therefore that the smaller sperms never attain maturity, whereas the large ones appear normal in every respect but size. They enter the sperm duct in a perfectly regular manner and there mingle with the sperms of the normal lobes.

MECISTORHINUS TRIPTERUS

The main points in which *Mecistorhinus tripterus* differs from *Mecistorhinus melanoleucus* involve the heteropycnosis of the autosomes and the behavior of the

sex chromosomes. So far as the diploid complement of chromosomes is concerned, there is no perceptible difference, both having an outstandingly large pair of autosomes and a Y that is the smallest of all the chromosomes. However, already in the spermatogonial resting phases there is a less extensive heteropycnosis in *Mecistorhinus tripterus*, for then as well as later there are never more than two heteropycnotic bodies (instead of from three to five). The spermatogenesis in the normal lobes is entirely orthodox. The heteropycnosis in the large autosomes is here lacking entirely and hence there is no association between them and the sex chromosomes (Fig. 45).

The lack of autosomal heteropycnosis obtains also in the harlequin lobe (Figs. 46 and 47). As a consequence, when the equatorial ring is formed the two sex



Mecistorhinus tripterus—Harlequin Lobe (except Fig. 45)

- FIGURE 45. Normal diakinesis; X and Y independent of autosomes (Feulgen).
 FIGURE 46. Early diplotene stage (Feulgen).
 FIGURE 47. Diakinesis; X and Y joined (Feulgen).
 FIGURE 48. Equatorial ring in formation; X and Y independent of autosomes (Feulgen).
 FIGURE 49. Anaphase I (Feulgen).
 FIGURE 50. Anaphase II—small cell (Feulgen).
 FIGURE 51. Anaphase II—large cell (Feulgen).

chromosomes lie in the approximate middle of the nuclear space instead of being carried to the periphery by the large autosomes (Fig. 48). The X and Y are at this time still in more or less intimate contact with each other and remain so until the first anaphase has begun.

Another difference lies in the size relations between the X and the Y. In *Mecistorhinus melanoleucus* the X is markedly larger than the Y, but this difference is less pronounced in *Mecistorhinus tripterus*. In the latter also, as in most other pentatomids, the sex chromosomes become less sharply outlined in the second division and therefore it then becomes difficult at times to distinguish between them (Fig. 50). As a consequence, in about 25 per cent of fifty cells it is not possible to decide whether the autosome follows the X and there is at least the possibility that in such

instances it accompanies the Y. Although there is thus no question that in this race too the association is between the X and the autosome in the great majority of cells, the case is not as clear-cut as it is in *Mecistorhinus melanoleucus*.

Mention should also be made of the fact that already in the first division, the first large autosome usually shows a median furrow (Fig. 49). If, as in *Mecistorhinus melanoleucus* this chromosome represents only one chromatid of one of the large univalents, the furrow is equivalent to a tertiary split. Whether that be correct or not, this split is not consummated even in the early spermatid, where the large autosome still maintains this appearance.

But in essence, the meiotic divisions of the two species are very much alike (Figs. 49-51, and 78). The difference in heteropycnosis does not affect the results and *Mecistorhinus tripterus* merely furnishes less decisive evidence anent the association between the X and the large autosome.

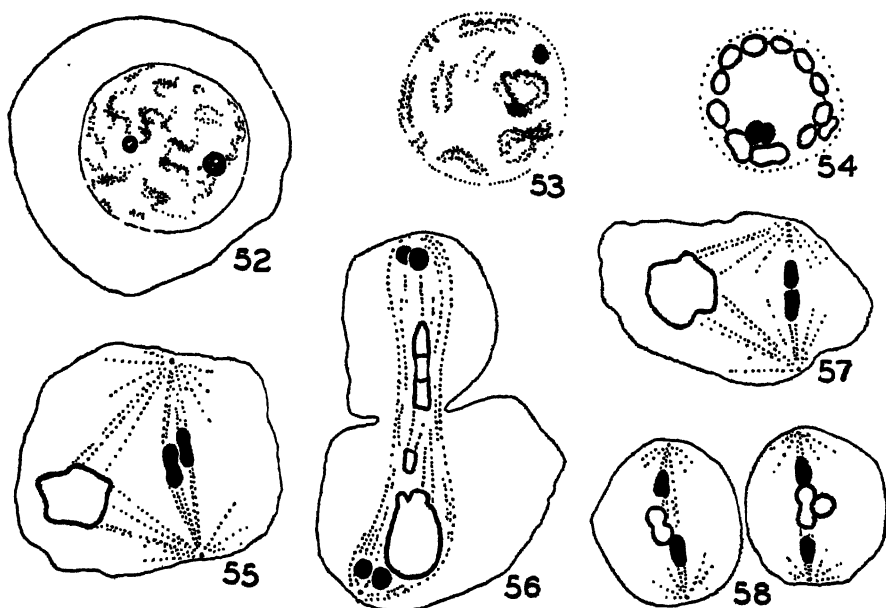
MECISTORHINUS SEPULCRALIS

The rather extensive material of this species from Brazil conforms closely in its general cytology to the Central American *Mecistorhinus* species. The diploid set is characterized by a very large pair of autosomes and the Y is again the smallest member of the set. The meiosis in the normal lobes follows an orthodox course although there are indications of heteropycnosis in a very restricted region of the two large autosomes.

This heteropycnosis is very much alike in normal and harlequin lobes. The spermatogonial resting phases may show as many as three or four very small heteropycnotic regions but more often only one larger one. In the meiotic prophase following the leptotene stage and up to diakinesis, it is difficult to recognize any heteropycnosis in the autosomes whereas both sex chromosomes are, as usual, heteropycnotic throughout. In the confused stage there are generally two heteropycnotic bodies, but whether these represent only the sex chromosomes or also certain autosomal regions it is not possible to say (Fig. 52). Only in mid-diakinesis do conditions allow a closer analysis. In the harlequin lobe, where we are dealing with univalents, both large autosomes then show heteropycnotic terminal regions, and these are joined so as to form closed rings as in *Mecistorhinus melanoleucus* (Fig. 53). Apparently this heteropycnosis is less extensive in the present species and this may account for the fact that the mutual attraction of such regions does not bring the two large univalents together in the "figure eight" formations that occur so often in the other forms. Probably for the same reason the association between the sex chromosomes and these autosomes is highly variable. Thus in some cells only one sex chromosome is joined to the heteropycnotic region of one univalent autosome while the other sex chromosome is free (Fig. 53). In late diakinesis such a condition is rare because, as in *Mecistorhinus tripterus*, there is then a strong tendency for the X and Y to come together. Hence the free sex chromosome often joins its attached mate and as a result both are carried into the equatorial ring of autosomes, just prior to the breakdown of the nuclear membrane (Fig. 54). In other cases the sex chromosomes may be entirely free and will then take a more or less central position in the nucleus at the time of the equatorial orientation of the autosomes.

But these various maneuvers that involve heteropycnotic attraction do not affect the course of the actual divisions in the harlequin lobe any more than in the normal

lobes. In the former the sex chromosomes at both metaphases become detached from the autosomal aggregate, which then is displaced toward the side of the cell just as in *Mecistorhinus tripterus* (Figs. 55 and 57). In *Mecistorhinus sepulcralis* however, the first anaphase shows a definitely greater tendency for other chromosomes to follow the first large autosome out of the aggregate. Although the second large autosome is included as rarely in the smaller second spermatocyte as in the preceding species, a smaller autosome sometimes succeeds in following the first autosome into this cell (in four out of forty cells) (Fig. 56). Apparently both these autosomes



Mecistorhinus sepulcralis—Harlequin Lobe

FIGURE 52. Confused stage (Feulgen).

FIGURE 53. Diakinesis stage (Feulgen).

FIGURE 54. Equatorial ring; X and Y associated with large autosomes (Feulgen).

FIGURE 55. Metaphase I (Hematoxylin).

FIGURE 56. Late anaphase I (Hematoxylin).

FIGURE 57. Metaphase II—large cell (Hematoxylin).

FIGURE 58. Anaphase II—small cell (Hematoxylin).

retain the "sticky" condition of the earlier aggregate, for when they reach the pole the smaller joins the larger one and adheres to it in almost any position in random fashion. In the second division, both follow the X to the pole in the majority of cases (Fig. 58).

But here too, as in the second division of *Mecistorhinus tripterus* the X and Y often show only a small size difference. In perfect side views they can nearly always be distinguished, but if the cell is viewed at a slight angle it at once becomes difficult to do so. Hence, though *Mecistorhinus sepulcralis* also shows a definite preferential association between the X and the large autosome (as well as the smaller, if present)

the possible occurrence of exceptions to the rule can no more be excluded than in the preceding form.

The division of the aggregate in the larger second spermatocyte occurs very much as it does in *Mecistorhinus tripterus*. The four main types of spermatids are therefore alike in all three species, comprising X + one autosome; Y; X + variable number of autosomes; Y + variable number of autosomes. The few exceptions in the present species are due to the occasional inclusion of a smaller autosome in the first named spermatid.

NEODINE MACRASPIS

This representative of another genus is in its cytological features almost a duplicate of *Mecistorhinus tripterus*. The cells and chromosomes of the harlequin lobe are slightly larger than those of the preceding forms. The large pair of autosomes however does not stand out quite so strikingly in its size as in the species of *Mecistorhinus* (Fig. 59). The normal meiosis is entirely orthodox (Figs. 60 to 63) and there is no heteropycnosis other than that of the sex chromosomes to complicate the prophase.

The harlequin lobe likewise lacks all autosomal heteropycnosis. The two sex chromosomes come into contact with each other during the confused period and this loose union is maintained through the diakinesis (Figs. 64 to 66) into the first metaphase (Fig. 67). They are entirely independent of the autosomes, and when the latter are marshalled to form their equatorial ring the X and Y are always found together in the middle of the nucleus (Fig. 66).

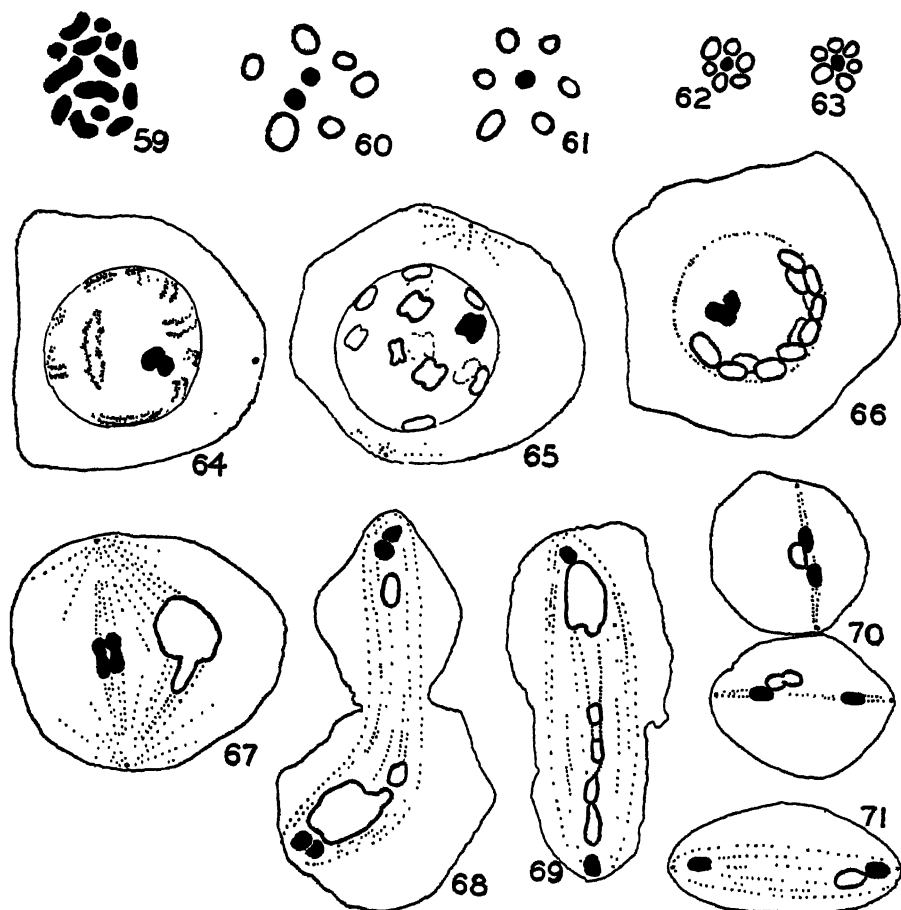
In the course of the first division, only one large autosome and no others join the X in the formation of the smaller second spermatocyte. This autosome may or may not exhibit the median tertiary split (Fig. 70).

The second division conforms to the general scheme described for the other species. The size difference between the X and Y is not as clear cut as in *Mecistorhinus melanoleucus*, but there is no doubt that in the great majority of cells, it is the X with which the single autosome is associated and not the Y (Figs. 70 and 71). The larger second spermatocyte divides irregularly and in the same manner as does the corresponding cell in *Mecistorhinus*.

In all essentials, therefore the meiosis in the harlequin lobe of *Neodine* parallels that of *Mecistorhinus*.

PLATYCARENUS NOTULATUS

In view of the uniformity in the occurrence of normal and harlequin lobes of the four preceding species, it comes somewhat as a surprise that in another species, *Platycarenum notulatus*, the conditions are entirely different. The structure of the testis departs radically from that of the other forms, there being only four instead of seven lobes, with a more compact instead of a serial arrangement. The diploid set of fourteen chromosomes is marked by no strikingly large pair of autosomes and only the exceptionally small size of the Y merits any notice. There is no harlequin lobe and the spermatogenesis takes a very orthodox course throughout the testis. The case thus serves warning that the cytological conditions are by no means uniform in the tribe Discocephalini as it is now constituted by the systematists.

*Neodine macraspis**Normal Lobe:*

- FIGURE 59. Spermatogonial metaphase (Hematoxylin).
 FIGURE 60. Metaphase I (Hematoxylin).
 FIGURE 61. Metaphase II (Hematoxylin).
 FIGURE 62. Telophase II; polar view with Y (Hematoxylin).
 FIGURE 63. Telophase II; polar view with X (Hematoxylin).

Harlequin Lobe:

- FIGURE 64. Early diakinesis; joined X and Y independent of autosomes (Hematoxylin).
 FIGURE 65. Late diakinesis; 12 univalents + joined XY (Hematoxylin).
 FIGURE 66. Formation of aggregate; joined X and Y separate (Hematoxylin).
 FIGURE 67. Early anaphase I (Hematoxylin).
 FIGURE 68. Telophase I (Hematoxylin).
 FIGURE 69. Telophase II—large cell (Hematoxylin).
 FIGURE 70. Anaphase II—small cell (Hematoxylin).
 FIGURE 71. Telophase II—small cell (Hematoxylin).

DISCUSSION

The most striking anomalies of the meiosis in the harlequin lobe of the *Discocephalini* may be summed up as follows: 1) Omission of pairing, 2) Clumping or aggregation of autosomes, 3) Preferential association between the X chromosome and a certain autosome.

Omission of pairing

The failure of the leptotene homologues to come together in synapsis has at present no cytological explanation. The only indication that conditions are not entirely orthodox at an earlier stage lies in the fact that the leptotene threads are less fine and attenuated than they are in normal lobes. In short they are not despiralized to the same extent as chromosomes that undergo the characteristic pairing reaction.

The further prophase behavior of the unpaired chromosomes is however of great interest. These univalents pass through all the meiotic phases that distinguish bivalents from the chromosomes of non-meiotic mitoses. They rapidly condense after the leptotene stage and a diplotene split makes its appearance; there follows a regular confused period; and the latter is in turn succeeded by an entirely typical series of diakinetid maneuvers. It would seem therefore that these characteristic prophase manifestations of meiosis are not at all conditioned by the phenomenon of pairing and, given certain basic conditions, the chromosomes run through the gamut of meiotic changes even when this most obvious feature of the meiotic prophase has been omitted.

Clumping and displacement of autosomes

The clumping or aggregation of autosomes which occurs suddenly just before the first metaphase and is not completely abandoned until the spermatid stage, is one of the most striking features of the harlequin lobe. It is only natural to seek some interrelation between this anomaly and the failure of synapsis that precedes it, but the connection is certainly not a direct one. Thus the omission of pairing in *Loxa* (Schrader, 1945b) is followed by no clumping in the first division (although there are indications of "stickiness" in the second). The most decisive evidence against such an interrelation is furnished by *Brachystethus* (Schrader, 1946). There the clumping of autosomes is even more persistent than in the *Discocephalini* but an omission of pairing obviously cannot be held responsible since the autosomes undergo normal synapsis and form typical tetrads.

The occurrence of autosomal heteropycnosis in the prophase of *Mecistorhinus melanoleucus* can also not be held responsible for the later aggregation of autosomes. In *Mecistorhinus tripterus* the clumping is just as marked as in the first named species, but there is no heteropycnosis in the autosomes at all. Indeed, despite the peculiar maneuvers that such heteropycnosis brings about especially during diakinesis, the meiotic divisions themselves are not perceptibly affected. They are essentially the same, whether autosomal heteropycnosis be present or not.

In all these considerations of the clumping phenomenon it must be remembered that the mitotic apparatus remains normal and cannot be held directly responsible for the anomalies. The justification for this conclusion lies not only in the fact that chromosomal spindle fibers are regularly formed between the centrioles and the

clumped autosomes, but also in the perfectly normal spindle relations of the two sex chromosomes.

It is the orthodox behavior of the sex chromosomes that may furnish a clue to the autosomal behavior. As usual, both the X and the Y chromosome are persistently heteropycnotic and at first metaphase are fully condensed. The autosomes however depart from their usual cycle of condensation and diffuseness. Already at prometaphase their precocious reaction to the polar forces would suggest a condition at variance with that which normally obtains—perhaps a more advanced state of condensation. Such abnormal timing is indicated more directly when the disintegration of the nuclear membrane finally occurs. The autosomes have then begun to reverse the normal sequence of development and when they undergo the clumping reaction they are in process of returning to the diffuse condition rather than attaining their final condensation. It is this untimely regression in nucleination (for there appears to be only a slight uncoiling) that probably underlies their clumping.

Such a hypothesis implies a close correlation between the state of condensation and the mutual reactions of chromosomes. It may be objected that during a normal prophase, when the chromosomes pass through all stages of condensation, no such clumping ever occurs. But it must be remembered that during the entire prophase a nuclear membrane is present and there can now be little doubt that this exerts the most far-reaching influence on chromosomal behavior (Schradler, 1941). Indeed when the membrane finally breaks down there is in normal cells also an immediate clumping of chromosomes which do not separate again until they reach final condensation shortly thereafter. And in the normal telophase, when the chromosomes have started to return to the diffuse condition, there is again a tendency to clump. It is only after the new nuclear membrane has been formed that the chromosomes separate and scatter once more. The so-called mutual repulsion of the chromosomes in these Pentatomidae and perhaps other forms as well thus appears to occur only when they are fully condensed or else are contained within a nuclear membrane.

It is possible that here also lies the key to the extrusion of the autosomal aggregate from the midregion of the spindle. As has already been said, the orthodox behavior of the sex chromosomes, in the same spindle proves that it is not in the spindle apparatus that an explanation is to be found. It is the autosomes and kinetochores that must be held responsible and the abnormality in their condition which results in clumping is probably also involved in their lateral displacement. That this aberrant condition is only temporary is indicated by the fact that there is a partial return to normal behavior in the second division. The aggregate of autosomes is then stretched between the two poles and, though still "sticky," many of its components become dissociated from each under the strain and again appear as individual chromosomes. By the time the spermatid stage has been reached their behavior appears to be normal and they scatter over the periphery of the nuclear membrane quite like the chromosomes of the neighboring lobes.

It is safe to assume that the establishment of the metaphase involves a set of precisely adjusted interactions between centers, chromosomes and kinetochores. That these interactions involve nothing more than a system of repulsions would seem to be very unlikely, and indeed the retention of the chromosomes within the compass of the equatorial plate almost demands forces of a positive nature. Whether these be contractile chromosomal spindle fibers or a zone of actual attraction in the equator or both, it is quite possible that an upset in the condition of one of the elements

involved might allow the forces of repulsion to gain the ascendancy.² On such a basis the regressive nucleination that is perhaps accompanied by a partial weakening of the kinetochores may well account for the expulsion of the autosomes in these *Discocephalini*.

The preferential segregation of one autosome

The special behavior of one of the autosomes and its association with the X chromosome has already been described. In analyzing its movements the following factors should be considered:

In the first division this chromosome shows no connection with either of the two sex chromosomes and its mitotic reactions are directed solely to the nearest pole. Its only distinction from the rest of the autosomes lies in a slightly greater condensation which is not very pronounced but may explain why it behaves more normally than they in this division. In the division of the smaller second spermatocyte this independence of the autosome is lost and to all intents and purposes it then becomes a satellite of the X chromosome.

The available evidence suggests that this chromosome represents a single chromatid of one of the large, univalent autosomes. It is probable that the two chromatids of this large univalent separate in a normal mitotic movement and that position in the aggregate determines which one frees itself and joins the two sex chromosomes at one pole. Its mate always moves to the opposite pole and with it goes the rest of the aggregate. The complications that attend these maneuvers arise primarily from the stickiness that tends to hold all autosomes together.

This provisional explanation of the peculiarities of the first division does not however throw much light on the division of the smaller second spermatocyte. The preferential segregation of the autosome is obviously a consequence of some interrelation with the X chromosome. To be sure, the two chromosomes are often connected by a Feulgen-positive strand or direct adhesion to each other. But before anaphase such a connection is sometimes also established between the autosome and the Y, and in still other cases there is no visible connection with either sex chromosome. Nevertheless, in the great majority of cases (about 95 per cent in *Mecistorrhinus melanoleucus*) it is the X with which the autosome is finally associated at telophase and not the Y. The evidence as it stands therefore admits of only one interpretation which has only a few parallels in the literature—the preferential segregation of the autosome is due to some kind of attraction between it and the X (or, much more unlikely, its repulsion by the Y). A directed movement is involved in any case and since the X behaves just as it would in a perfectly normal cell it is the autosome itself which is primarily accountable.

Evolutionary aspects

It is a matter of surprise that so striking a feature as is comprised by the harlequin lobe should have escaped the notice of cytologists for so many years. To be sure it has so far been encountered only in the hemipteran family of Pentatomidae, but that particular group of insects has been subjected to more intense cytological study than almost any other comparable group except the orthopteran family of

² In a short article that has just come to hand, Östergren (Bot. Notiser, 1945) suggests that if the spindle is a tactoid such an interplay of forces may well be involved.

Acrididae. Work on some 70 species has been published and among the investigators have been such outstanding cytologists as Montgomery, Wilson, and Geitler. To now discover harlequin lobes in some eight genera and eleven species, widely scattered through the family, can hardly mean that their occurrence has simply been overlooked hitherto. We must seek an explanation elsewhere and in such a quest one cannot fail to be struck by one aspect of the situation. It so happens that despite the great number of species investigated all cytological work has been confined to Pentatomidae from the Palaearctic and Nearctic regions. The rich pentatomid fauna of the Oriental, Australasian, and Ethiopian regions has never been touched by cytologists, and only recently have Neotropical species been investigated. It is among the latter that I have encountered all the species that carry harlequin lobes and the question might well be asked whether the occurrence is not correlated with a tropical habitat. But that is not to say that all tropical Pentatomidae are characterized by such lobes for my own investigations have shown that a number of species of such genera as *Alcaeorrhynchus*, *Arocera*, *Edessa*, etc., do not possess it. Nevertheless the correlation is sufficiently striking to make an inquiry into the effects of various factors in tropical conditions on the development of reproductive organs well worth while.

The occurrence of harlequin lobes in four different species of the tribe Discocephalini collected in localities as far apart as Costa Rica and southern Brazil (about 5400 km. or 3350 miles) would seem to justify the conclusion that such a feature was present in the ancestral species of the tribe. Its absence in a fifth, a species of *Platycarenum*, might be attributable to loss in the course of evolution. But harlequin lobes are found also in some members of entirely different tribes such as the *Halyini* (unpublished) and the *Pentatomini*, and in such a remote form as *Brachystethus* (Schrader, 1946) its cytological character is obviously very similar to that of the *Discocephalini*. On the basis of our original reasoning we would thus arrive at the conclusion that a harlequin lobe characterized not only the first discocephalinid but was present already in the more remote ancestor of the whole family of Pentatomidae. Its sporadic occurrence at the present time may therefore be representative of evolutionary survivals that are possible only under certain conditions, though of course it is possible that the genetic basis for the harlequin lobe has existed for a long time but that this can express itself only under certain rather limited conditions. In either case, the deciding factor may well lie in the environment which happens to be more often favorable in tropical than in temperate regions.

Since the sperms of the harlequin lobe are ordinarily nonfunctional in the hereditary sense, it is obvious that in such species there is a very great loss of gametes. Natural selection might confidently be expected to eliminate so wasteful a development unless it also confers certain advantages that compensate for the loss of functional sperms. It has been suggested (Schrader, 1946) that these advantages may lie in the nucleoproteins that are carried into the eggs by supernumerary sperms. Polyspermy is of extremely common occurrence in the fertilization of insect eggs and it is probable that the nucleoproteins introduced by the extra sperms are utilized by the growing embryo. The sperms from the harlequin lobe are usually much larger than normal sperms and hence would contribute proportionately larger amounts of nucleoproteins. Species with harlequin lobes may therefore hold an advantage over those that lack them—an advantage that takes the form of an increase in materials that are of special value for the developing embryo. Certain it is that so

prosperous a species as *Mecistorhinus tripterus* which has a distribution from Vera Cruz, Mexico, to São Paulo, Brazil, has found the possession of a harlequin lobe no disadvantage.

The sporadic and yet widespread occurrence of harlequin lobes among the species of Pentatomidae argues strongly that we are dealing with a basic condition. The problem of why a certain lobe in the testis always takes a special course in its development may in the end be no different from that involved in such a case as the development of diverse types of digestive glands from the embryonic gut. In other words, we are confronted here, as well as there, by the old question of differentiation.

SUMMARY

1. In the testes of four species of the pentatomid tribe Discocephalini, the fifth lobe always shows a special type of meiosis.
2. There is no pairing in this lobe.
3. Just prior to metaphase I all the autosomes aggregate in one clump. The sex chromosomes behave quite normally through both meiotic divisions.
4. In the first division the X and Y divide equationally as in normal cells. One large autosome becomes dissociated from the aggregate and reaches the pole opposite to that attained by the rest of the aggregate.
5. A large and a small second spermatocyte result from this first division. In the small one, the single autosome goes to the same pole as the X in the great majority of second divisions.
6. In the division of the larger second spermatocyte, the aggregate is irregularly pulled into two groups.
7. Four main types of spermatids result: X + variable number of autosomes; Y + variable number of autosomes; X + one large autosome; Y. The last two types probably never reach the mature sperm stage.
8. It is pointed out that so firmly established a feature as this seemingly wasteful development in the fifth or "harlequin" lobe must have some compensatory advantage, and its cytological and evolutionary significance are discussed.

All figures are photographs from harlequin lobe; Figs. 72 to 77 inclusive from *Mecistorhinus melanoleucus*; Fig. 78 from *Mecistorhinus tripterus*.

FIGURE 72. Formation of equatorial ring (Hematoxylin).

FIGURE 73. Early metaphase I with the two sex chromosomes close together in the middle and the autosomal aggregate displaced to one side (Hematoxylin). Shown in two of the cells.

FIGURE 74. Beginning of anaphase I; X and Y in middle, large autosome beginning to protrude from displaced aggregate (Gentian violet).

FIGURE 75. Late anaphase I, photographed at two different levels; large autosome leaving aggregate, and an X and Y approaching each pole (Gentian violet).

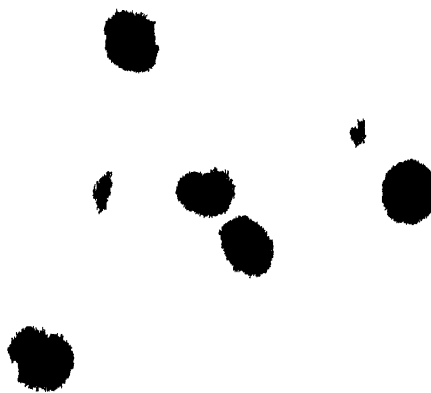
FIGURE 76. Anaphase II (large cell); aggregate displaced to one side and X and Y separating to opposite poles (X to lower pole) (Hematoxylin).

FIGURE 77. Anaphase II (small cell); X with autosome going to upper pole and Y to lower pole (Hematoxylin).

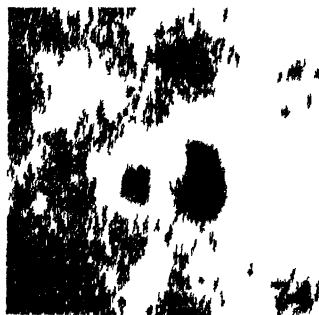
FIGURE 78. Telophase II (large cell); aggregate becoming dissociated between separating X (lower pole) and Y (upper pole) (Feulgen).



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THE EFFECT OF THE ADULT ANTERIOR PITUITARY HORMONE ON THE TADPOLES AND THE IMMATURE MALE FROGS OF THE BULLFROG, *RANA CATESBIANA*

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INTRODUCTION

The interrelationship of the anterior pituitary hormone and the gonads of vertebrates has been well established. There is evidence that the amphibian gonads, under the influence of the anterior pituitary hormone stimulation, will either respond immediately (in mature animals) by the liberation of mature gametes or will differentiate (if immature) in much the same manner as they do during the breeding season, with the exception that these artificially stimulated changes are accomplished at a much faster rate. This statement is supported by the work of Riddle and Flemion (1928) on the dove; Houssay and Lascano-Gonzalez (1929) on the South American toad; Herre and Raiviel (1939) on larvae of Triton; Burns (1930) and Burns and Buyse (1934) on *Amblystoma*; Evans (1935) on the lizard; Forbes (1937) on the alligator; Rugh (1937, 1939, and 1941) on frogs of different genera; Puckett (1939) on the bullfrog tadpole, and Glass and Rugh (1944) on the mature frog, *Rana pipiens*. In this latter paper it was shown that while there are seasonal changes in the frog testis, indicating cyclical maturation, the gonad will respond at any time of the year to frog anterior pituitary hormone stimulation by the liberation of any mature spermatozoa which are present, and that frequently (especially during active maturation of August) the seminiferous tubules will also contain other maturation stages. Recently Schreiber and Rugh (1945) have studied the response of the testes of recently metamorphosed *Rana pipiens* to the pituitary hormone and to estradiol-benzoate.

It was the purpose of this investigation to determine the effect of the anterior pituitary hormone of the adult frog on the gonad of the pre- and the post-metamorphosing male bullfrog. The testis can be differentiated from the ovary macroscopically even before tail absorption in the tadpole of *Rana catesbiana*.

MATERIALS AND METHODS

The tadpoles of *Rana catesbiana* were collected in the vicinity of Woods Hole, Mass., and were used immediately. During the month of August all stages of pre- and post-metamorphosis are available in great abundance. These tadpoles are in their second year of growth, and generally go through the stages of metamorphosis rather rapidly, depending upon the water temperature and the amount of food available. Since these tadpoles were well fed in their natural habitat, there was no attempt to feed them during the short period of the experiment, three to four days.

Tadpoles were selected in five stages:

- a. Pre-metamorphic stage where there were four well-developed appendages and a full-length tail fin.
- b. Post-metamorphic stage where the body length was less than 45 mm. and the tail had just been absorbed, but could be identified as a stub. These frogs had just shifted to lung breathing.
- c. Frogs of body length of 65 mm.
- d. Frogs of body length of 95 mm.
- e. Mature, full-grown frogs whose body length was at least 120 mm.

These five stages represented a graded series from pre-metamorphosis to the sexually mature adult male, covering a period of normal development ranging from August of one year to the summer of the following year, at least.

The experimental animals were injected within a few hours or, at the most, within 24 hours of collection in the ponds. In most cases the right testis was removed and fixed and the animal was allowed to recover from the gonadectomy and the abdominal wound was permitted to heal for 24 hours. Then each animal was injected with two whole anterior pituitary glands from sexually mature, pre-ovulating female bullfrogs. This is a dose known to be adequate to induce sexual activity in adults (Rugh, 1943). The hormone was injected whole, through a large bore hypodermic needle, into the body cavity, along with about 2 cc. of distilled water. The animal was then placed in a battery jar with a small amount of water and kept at laboratory temperatures. Forty-eight hours later each animal was sacrificed and the remaining (left) testis was removed and fixed in a manner identical with that for the right testis previously removed. In all instances the gonad was removed without direct handling for it has been shown (Rugh, 1939; 1941) that mere manipulation of the living testis will often alter the internal picture. The fat bodies were used as "handles" in excision and removal of the testis. The whole organ was placed in Bouin's solution for 24 hours; slowly run up through to 70 per cent alcohol where the yellow picric stain was removed by the addition of 5 per cent by volume of concentrated NH_4OH for several half-hour changes; dehydration was completed in higher alcohols; clearing in xylol; embedding in a mixture of 90 per cent paraffin (58°MP), 5 per cent bayberry wax, and 5 per cent beeswax. The gonads were sectioned at 8 microns and were stained with Heidenhain's iron-haematoxylin with counterstain.

The photographs were taken on Microcopy film (35 mm.) by means of a Microfilm adapter.

OBSERVATIONS AND EXPERIMENTAL DATA

Pre-metamorphic stage

The testis of the pre-metamorphic bullfrog tadpole, which stage possesses all four appendages and a tail, appears as a solid yellowish organ associated with the adjacent reddish kidney by means of the double-layered mesorchium. In section (Figs. 1 and 9) the gonad does not show well-formed seminiferous tubules partly because there is an abundance of interstitial tissue and there are no late maturation stages and no lumina. Scattered around the periphery of the developing seminiferous tubules are found huge primary spermatocytes which seem to be adherent to the base-

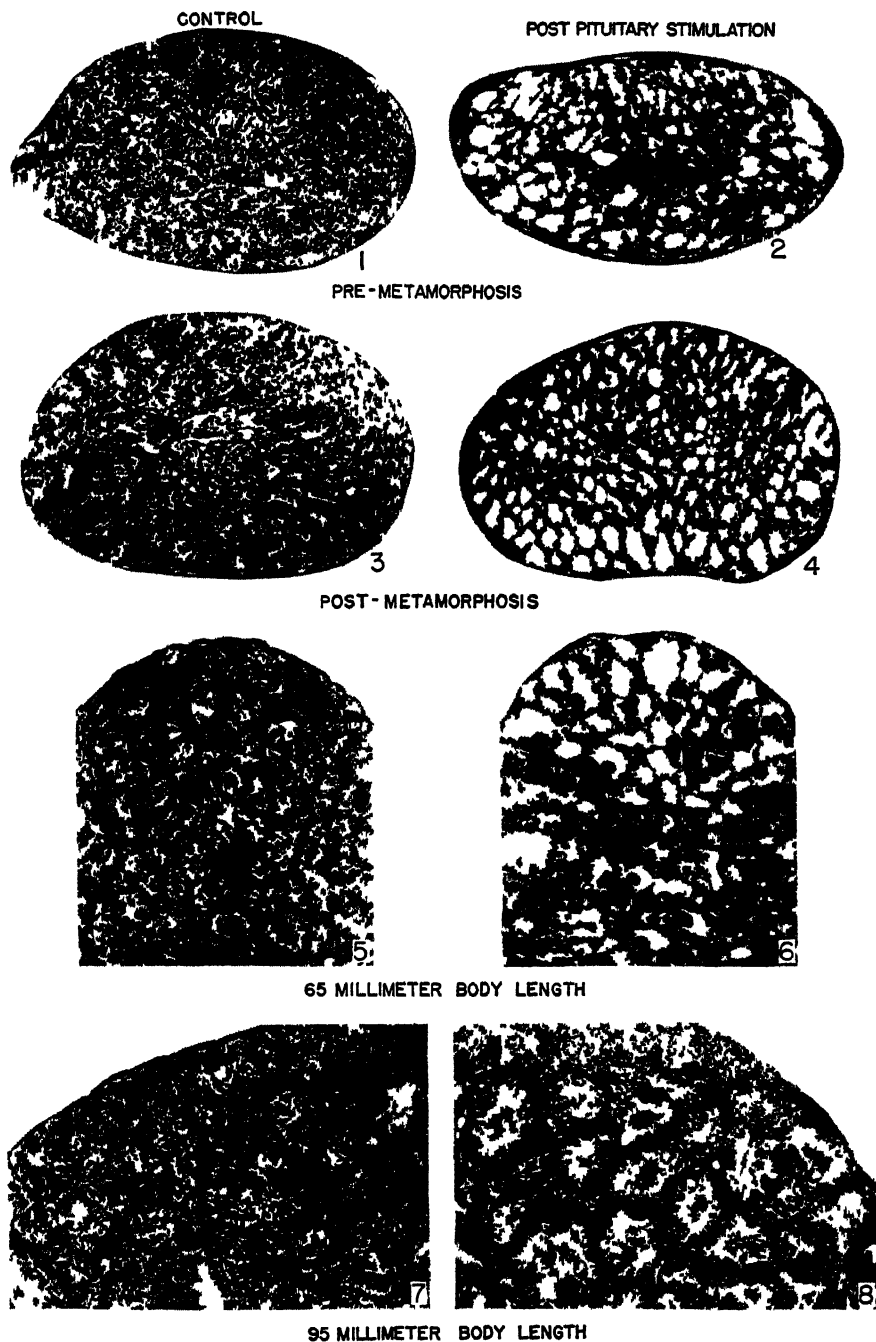


PLATE I Effect of anterior pituitary hormone on testis of immature bullfrog *R. catesbeiana*

ment membrane of the tubule. There are also oval-shaped darkly staining nuclei of the spermatogonia, and occasionally there may be seen (toward the region of the future lumen) some secondary spermatocytes. The average length of these testes is 2.18 mm. and the average diameter is 0.80 mm.

When the pre-metamorphic tadpole is subjected to the anterior pituitary hormone (by coelomic injection) the most obvious change in the testis is the vacuolization of the forming seminiferous tubules (Figs. 2 and 10) so that the entire gonad takes on a sponge-like appearance. The peripheral region of the gonad is always the first to be affected, and the most vacuolated. Under high power magnification it is noted that the only cells remaining within the seminiferous tubules are those attached to or an integral part of the basement membrane. The other cells which had previously occluded the lumen will be seen in the collecting tubules and the vasa efferentia, indicating that they were enroute through the reproductive tract at the time the gonad was fixed. One gets the impression that the gonad was flushed out of all loosely attached cellular components.

The size variations of the control and the experimental gonads were not appreciably different.

Post-metamorphic stage, body length less than 45 mm.

In this stage the gonad itself has enlarged considerably so that the maximum length of the testis is about 2.6 mm. and the maximum diameter is about 1.8 mm. The shape of the gonad is also changed, since its length increased about 40 per cent while its diameter increased about 125 per cent.

In the control testis (Figs. 3 and 11) there is some evidence of the forming seminiferous tubules, and maturation stages from spermatogonia to mature spermatozoa are seen. This indicates that coincidental with the drastic changes of metamorphosis the bullfrog tadpole's testis first acquires structurally mature spermatozoa. Whether or not these spermatozoa are functional has not yet been determined. Clusters of cells are seen, all in the same stage of maturation, with the progressively advanced stages more nearly in the center of the seminiferous tubules.

Here again, when the testis is subjected to the anterior pituitary hormone of the adult, there is a liberation from the tubules of all freely associated cells (Figs. 4 and 12) so that the post-stimulated gonad has much the same appearance as that of the experimental testes of the pre-metamorphic stage (Figs. 2 and 10). There remain only the spermatogonia, primary spermatocytes, and the few interstitial cells. The majority of cells found within the collecting tubules and the vasa efferentia (Fig. 17) are secondary spermatocytes.

65 mm. body length stage

By this stage of development the testis has acquired its adult proportions and measures about 3.35 mm. in length and 2.0 mm. in diameter. The surface has become lobulated due to the further development of the seminiferous tubules about the periphery. Nests of maturing cells are seen (Figs. 5 and 13) representing all stages from the primary spermatocytes to the spermatids and mature spermatozoa. The early maturation figures are in great abundance with the pachytene groups the most prominent (Fig. 13). It is at this stage that the cysts or nesting groups of cells become distinct from each other, demarking those clusters of cells, all of which originate from the same spermatogonium. With the enlargement of the seminiferous

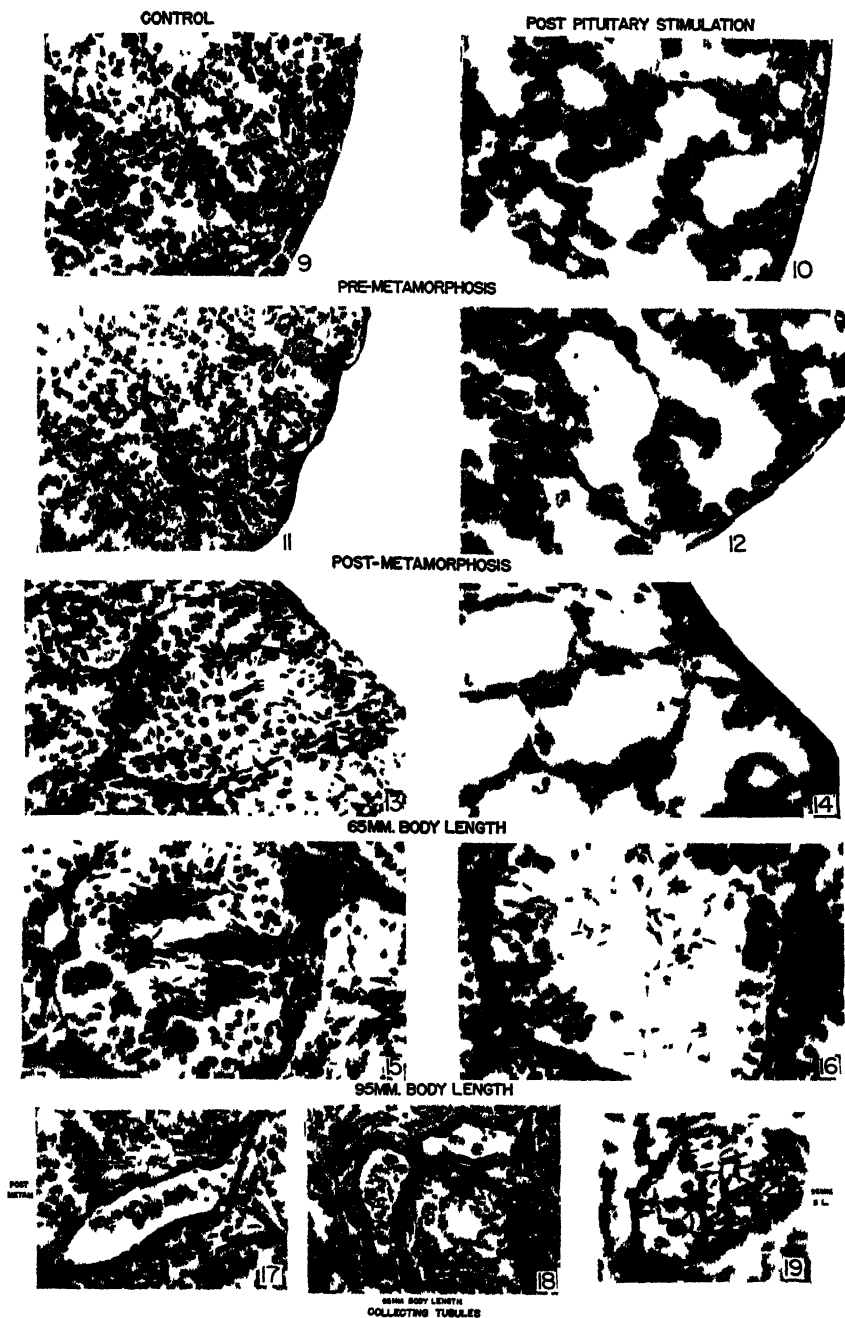


PLATE II. Effect of anterior pituitary hormone on seminiferous and collecting tubules *R. catesbeiana*.

tubule there appears to be a compensatory reduction in the amount of interstitial tissue, due, no doubt, to the stretching of this tissue in the general growth of the gonad.

Upon stimulation of this testis by the anterior pituitary hormone of the same species, there is a loss from the gonad of all or most of its freely associated cells and cell groups (Figs. 6 and 14). The gonad seems to have its peripheral tubules emptied the more completely, and, upon closer examination, it is noted that often there remain only the scattered primary spermatocytes and spermatogonia. The primary spermatocytes are fewer in relative number than in the previous stage studied. This is probably due to the fact that some of these cells have been utilized in the production of the maturation nests of cells, some of which were liberated under hormone stimulation. The maturation stages which are missing from the seminiferous tubules are found within the collecting tubules (Fig. 18) and the vasa efferentia leading from the testis to the kidney.

95 mm. body length stage

Except for size, this testis has the histological appearance of the adult gonad. The length is about 5.1 mm. while the diameter is about 2.5 mm. The seminiferous tubules are fully formed and distinct from each other, and the tunica albuginea has become considerably thickened.

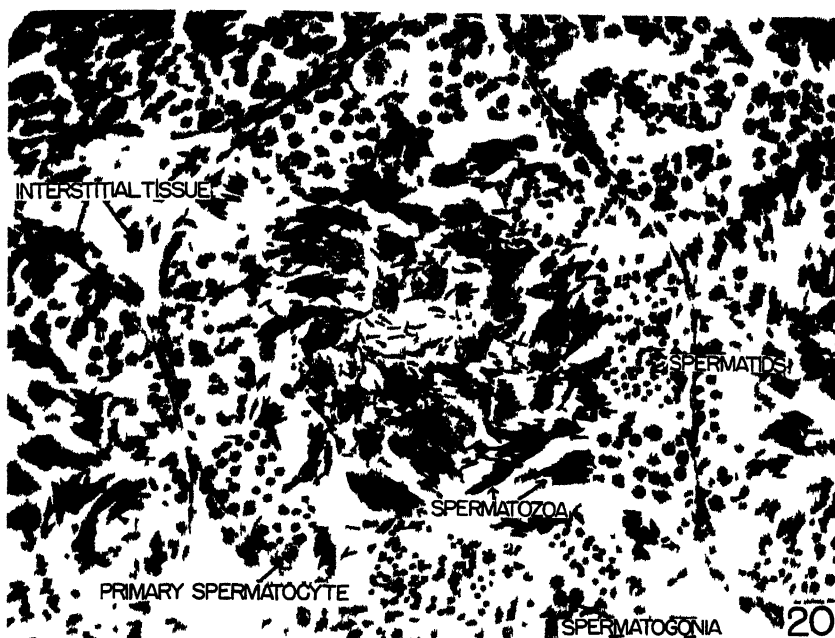
The cysts or nests of cells in the seminiferous tubule include the majority of cells in the more advanced stages of maturation, with abundant fully formed spermatozoa (Figs. 7 and 15). There are many nests of spermatids, and of secondary spermatocytes, but it is with some difficulty that one locates primary spermatocytes.

As a result of anterior pituitary hormone stimulation the lumina of this testis are always enlarged but there is not as complete evacuation of the various cell types as in the previous stage of development studied. That is, some of the secondary spermatocytes and spermatids remain unaffected by the hormone (Figs. 8 and 16), but these same cell types are found within the collecting tubules (Fig. 19) and in the related vasa efferentia.

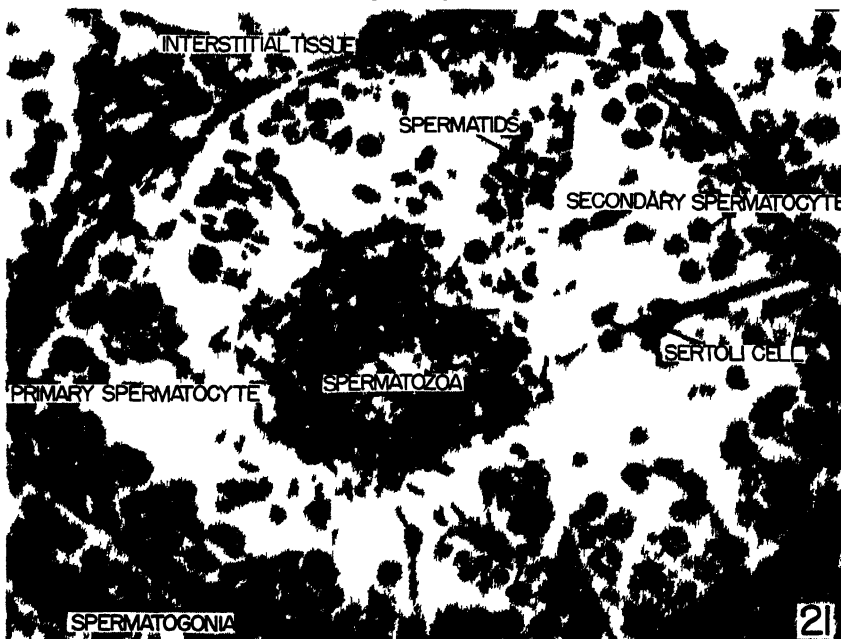
While the obvious response of the testis as a whole is similar to that of earlier stages of development, there seems to be a greater resistance of many of the cell types, particularly the earlier stages, to evacuation.

The mature bullfrog, body length of more than 120 mm.

The testis of the mature bullfrog measures about 9.8 mm. in length and 5.4 mm. in diameter, being oval in shape and yellowish-white in color. The interstitial tissue is stretched until it remains only as a thin line between seminiferous tubules. Depending upon the season of the year the maturation picture will vary but at the time of this experiment (August) there is normally heightened sexual activity which means abundant mature spermatozoa and abundant reserve cells ready to proceed through maturation, immediately following amplexus and elimination of the mature gametes. Such a picture is seen in Figure 20. It is difficult to locate any spermatogonia or primary spermatocytes, although they are present, due to the abundance of later maturation stages. The mature spermatozoa are in clusters with their heads buried in the cytoplasm of Sertoli cells, and with their tails filling the lumina of the seminiferous tubules. Such a testis is excellent for the study of all of the



CONTROL



POST PITUITARY STIMULATION

steps in maturation, but, in contrast with the younger frogs there are relatively few of the early maturation stages, i.e., spermatogonia.

Upon stimulation by anterior pituitary hormone the picture becomes somewhat clearer, for the seminiferous tubules are emptied of the mature spermatozoa and an occasional spermatid, and consequently the other stages of maturation within the seminiferous tubules become visible (Fig. 21). The collecting tubules and the related vasa efferentia, however, contain almost exclusively the mature spermatozoa indicating a resistance to liberation of the earlier maturation stages. This resistance was not in evidence with the gonads of younger stages. Sperm release is independent of any overt sex behavior on the part of the male, being a direct response to the anterior pituitary hormone stimulation.

DISCUSSION

The presence of smooth muscle fibers in the ovaries of the frog has been demonstrated (Rugh, 1935) and the contraction of the lobes of the ovary has been seen in highly magnified moving pictures. It has further been demonstrated that the anterior pituitary hormone acts not directly upon the gonad but by way of either or both the nervous and the circulatory systems (Rugh, 1935 and Samartino and Rugh, 1945). This hormone, further, has been shown to stimulate other secondary sexual characters such as the thumb pad of the adult (Glass and Rugh, 1944) and the Mullerian ducts of the immature *Rana pipiens* (Schreiber and Rugh, 1945). In view of these and other findings, it is now clear that upon injection of the anterior pituitary hormone of the frog into the body cavity of the frog, supplementing its own glandular secretions, both the primary and secondary sexual characters are stimulated and the ovary (at least) utilizes its smooth muscle elements to free its follicles of their enclosed eggs.

Puckett (1939) and previously Swingle (1921 and 1926) found a race of bullfrog tadpoles in which there were so-called undifferentiated gonads, particularly in the male, known as protestes. These protestes reacted to daily injections of various mammalian hormones (pituitary and gonadal) by showing precocious development and differentiation, particularly when male sex and pituitary hormones were injected simultaneously. There was no influence on the differentiation of the gonads with regards to sex, but rather the pituitary treatment determined the rate at which the gonads grew. The variety of *Rana catesbiana* used in the experiments reported in this paper did not show the stage of undifferentiated gonads described by Puckett, but they did show a decided response to the amphibian pituitary alone. Adult female frogs have been found to be almost totally resistant to mammalian pituitary extracts (Rugh, 1935 and 1942), while the closely related toads and salamanders would respond readily.

Recently Schreiber and Rugh (1945) treated metamorphosing *Rana pipiens* with the adult frog anterior pituitary hormone and also with the female hormone estradiol benzoate. These hormones were used independently and it was demonstrated that the pituitary hormone acted on the gonad while the estradiol acted upon the gonaducts (oviducts and Mullerian ducts). It is therefore evident that the gonad, whether immature or mature, will respond to anterior pituitary hormone stimulation if the frog is treated with adult amphibian pituitary glands.

The period of this experiment, i.e., 48 hours, was too short to allow demonstra-

tion of any growth response of the gonads but there was immediate and drastic response at all stages, whether spermatozoa were present or not, by the elimination of cellular elements from the forming seminiferous tubules. Since there is experimental proof of smooth muscle presence and activity in the frog ovary, and since the post-stimulation picture of the frog testis indicates a greater response at the periphery, it is suggested that here too there may be pituitary stimulation of smooth muscle elements within and surrounding the testis which causes the evacuation of cellular elements. Even if there were evidence of selective action of the pituitary hormone on a specific cell type such an hypothesis would have no real precedent. We are dealing with phases in the maturation of one cell type and, in the immature gonad, all steps in maturation are found within the collecting tubules and the vasa efferentia.

SUMMARY AND CONCLUSIONS

1. Bullfrog tadpoles and immature male frogs of body lengths of 45, 65, 95, and mature bullfrogs of 120 mm. body lengths, possessing normally differentiating gonads, were treated with anterior pituitary glands from adult frogs of the same species.

2. Scattered spermatozoa normally appear shortly after metamorphosis but by the stage of 65 mm. body length there are relatively abundant structurally mature spermatozoa.

3. The short duration of the experiment, i.e., 72-96 hours, did not allow for any demonstrable growth response of the testis to the anterior pituitary hormone.

4. Even before mature spermatozoa appear, or there is any evidence of a lumen within the seminiferous tubule, the testis of the immature bullfrog reacts to the anuran anterior pituitary hormone by evacuation of cellular elements of the forming seminiferous tubule, and by the formation of exaggerated lumina.

5. Cellular elements, found after pituitary stimulation within the collecting tubules and the vasa efferentia, consist of all stages in maturation except the spermatogonia. Whole cysts of similar cells have been seen, indicating rather violent evacuation of the gonad.

6. The more mature the testis the more resistant are the earlier maturation stages to anterior pituitary hormone release from the gonad, so that in the collecting tubules of the newly metamorphosed bullfrog testis there may be found all stages from spermatocytes to spermatozoa but in the tubules of sexually mature (adult) males there are rarely any cells except spermatozoa.

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THE BIOLOGICAL BULLETIN

PUBLISHED BY THE MARINE BIOLOGICAL LABORATORY

THE MARINE BIOLOGICAL LABORATORY

FORTY-EIGHTH REPORT, FOR THE YEAR 1945—FIFTY-EIGHTH YEAR

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II. ACT OF INCORPORATION

No. 3170

COMMONWEALTH OF MASSACHUSETTS

Be It Known, That whereas Alpheus Hyatt, William Sanford Stevens, William T. Sedgwick, Edward G. Gardiner, Susan Minns, Charles Sedgwick Minot, Samuel Wells, William G. Farlow, Anna D. Phillips, and B. H. Van Vleck have associated themselves with the intention of forming a Corporation under the name of the Marine Biological Laboratory, for the purpose of establishing and maintaining a laboratory or station for

scientific study and investigation, and a school for instruction in biology and natural history, and have complied with the provisions of the statutes of this Commonwealth in such case made and provided, as appears from the certificate of the President, Treasurer, and Trustees of said Corporation, duly approved by the Commissioner of Corporations, and recorded in this office;

Now, therefore, I, HENRY B. PIERCE, Secretary of the Commonwealth of Massachusetts, *do hereby certify* that said A. Hyatt, W. S. Stevens, W. T. Sedgwick, E. G. Gardner, S. Minns, C. S. Minot, S. Wells, W. G. Farlow, A. D. Phillips, and B. H. Van Vleck, their associates and successors, are legally organized and established as, and are hereby made, an existing Corporation, under the name of the MARINE BIOLOGICAL LABORATORY, with the powers, rights, and privileges, and subject to the limitations, duties, and restrictions, which by law appertain thereto.

Witness my official signature hereunto subscribed, and the seal of the Commonwealth of Massachusetts hereunto affixed, this twentieth day of March, in the year of our Lord One Thousand Eight Hundred and Eighty-Eight.

[SEAL]

HENRY B. PIERCE,
Secretary of the Commonwealth.

III. BY-LAWS OF THE CORPORATION OF THE MARINE BIOLOGICAL LABORATORY

I. The members of the Corporation shall consist of persons elected by the Board of Trustees.

II. The officers of the Corporation shall consist of a President, Vice President, Director, Treasurer, and Clerk.

III. The Annual Meeting of the members shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Massachusetts, at 11:30 A.M., and at such meeting the members shall choose by ballot a Treasurer and a Clerk to serve one year, and eight Trustees to serve four years; and shall transact such other business as may properly come before the meeting. Special meetings of the members may be called by the Trustees to be held at such time and place as may be designated.

IV. Twenty-five members shall constitute a quorum at any meeting.

V. Any member in good standing may vote at any meeting, either in person or by proxy duly executed.

VI. Inasmuch as the time and place of the Annual Meeting of members are fixed by these By-laws, no notice of the Annual Meeting need be given. Notice of any special meeting of members, however, shall be given by the Clerk by mailing notice of the time and place and purpose of such meeting, at least fifteen (15) days before such meeting, to each member at his or her address as shown on the records of the Corporation.

VII. The Annual Meeting of the Trustees shall be held on the second Tuesday in August in each year, at the Laboratory in Woods Hole, Mass., at 10 A.M. Special meetings of the Trustees shall be called by the President, or by any seven Trustees, to be held at such time and place as may be designated, and the Secretary shall give notice thereof by written or printed notice, mailed to each Trustee at his address as shown on the records of the Corporation, at least one (1) week before the meeting. At such special meeting only matters stated in the notice shall be considered. Seven Trustees of those eligible to vote shall constitute a quorum for the transaction of business at any meeting.

VIII. There shall be three groups of Trustees:

(A) Thirty-two Trustees chosen by the Corporation, divided into four classes, each to serve four years; and in addition there shall be two groups of Trustees as follows:

(B) Trustees *ex officio*, who shall be the President and Vice President of the Corporation, the Director of the Laboratory, the Associate Director, the Treasurer, and the Clerk;

(C) Trustees *Emeritus*, who shall be elected from the Trustees by the Corporation. Any regular Trustee who has attained the age of seventy years shall continue to serve as Trustee until the next Annual Meeting of the Corporation, whereupon his office as regular Trustee shall become vacant and be filled by election by the Corporation and he shall become eligible for election as Trustee *Emeritus* for life. The Trustees *ex officio* and *Emeritus* shall have all the rights of the Trustees except that Trustees *Emeritus* shall not have the right to vote.

The Trustees and officers shall hold their respective offices until their successors are chosen and have qualified in their stead.

IX. The Trustees shall have the control and management of the affairs of the Corporation; they shall elect a President of the Corporation who shall also be Chairman of the Board of Trustees; and shall also elect a Vice President of the Corporation who shall also be the Vice Chairman of the Board of Trustees; they shall appoint a Director of the Laboratory; and they may choose such other officers and agents as they may think best; they may fix the compensation and define the duties of all the officers and agents; and may remove them, or any of them, except those chosen by the members, at any time; they may fill vacancies occurring in any manner in their own number or in any of the offices. The Board of Trustees shall have the power to choose an Executive Committee from their own number, and to delegate to such Committee such of their own powers as they may deem expedient. They shall from time to time elect members to the Corporation upon such terms and conditions as they may think best.

X. Any person interested in the Laboratory may be elected by the Trustees to a group to be known as Associates of the Marine Biological Laboratory.

XI. The consent of every Trustee shall be necessary to dissolution of the Marine Biological Laboratory. In case of dissolution, the property shall be disposed of in such manner and upon such terms as shall be determined by the affirmative vote of two-thirds of the Board of Trustees.

XII. The account of the Treasurer shall be audited annually by a certified public accountant.

XIII. These By-laws may be altered at any meeting of the Trustees, provided that the notice of such meeting shall state that an alteration of the By-laws will be acted upon.

IV. THE REPORT OF THE TREASURER

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen:

Herewith is my report as Treasurer of the Marine Biological Laboratory for the year 1945.

The accounts have been audited by Messrs. Seamans, Stetson, and Tuttle, certified public accountants. A copy of their report is on file at the Laboratory and inspection of it by members of the Corporation will be welcomed.

The principal summaries of their report—The Balance Sheet, Statement of Income and Expense, and Current Surplus Account—are appended hereto as Exhibits A, B, and C.

The following are some general statements and observations based on the detailed reports:

*I. Assets**1. Endowment Assets*

As of December 31, 1945, the total book value of all the Endowment Assets, including the Scholarship Funds, was \$966,772.16, a loss for the year of \$17,128.41. The decline was due, as in the last two years, to losses on the mortgage participations on New York City realty held in the Trust Funds.

At the end of the year \$831,993.01 was invested in marketable securities (bonds, preferred stocks and common stocks) with a market value of \$910,162.31. \$125,753.85 was invested in mortgage participations on New York City real estate. \$9,025.30 was in uninvested principal cash.

The Treasurer's estimate of the actual value of the \$125,753.85 in mortgage notes and participations held on December 31 is \$85,750.00. With the market value of \$910,162.31 on marketable securities and the \$9,025.30 in cash this makes a total current valuation of \$1,004,937.61 compared with total book value of \$966,772.16.

The increase for the year in market values, \$75,454.79, is largely due to the rise in common stock prices.

2. Plant Assets

There were no changes of any consequence in Plant Assets during the year. The Reserve Fund was increased nearly \$10,000.00 to a total of \$26,830.71 by the transfer of the Crane Co. dividends, part of the General Biological Supply House dividends and other items of current income.

3. Current Assets

The total of current assets increased \$10,730.68 during 1945 to a total of \$212,970.35. Current Liabilities at the end of the year were \$2,754.70. Current Surplus increased \$12,277.94 to a total of \$196,337.90.

II. Income and Expenditures

The return to more normal operations for the Laboratory last summer resulted in larger totals for both income and expense items. Total income was \$182,818.23, total expense including depreciation reserves of \$26,968.12 was \$173,044.95, giving a net surplus for the year of \$9,773.28.

EXHIBIT A

MARINE BIOLOGICAL LABORATORY BALANCE SHEET, DECEMBER 31, 1945

Assets

Endowment Assets and Equities:

Securities and Cash in Hands of Central Hanover Bank and Trust Company, New York, Trustee	\$ 950,130.04
Securities and Cash in Minor Funds	16,642.12

\$ 966,772.16

REPORT OF THE TREASURER

7

Plant Assets:

Land	\$ 111,425.38	
Buildings	1,326,345.54	
Equipment	187,837.87	
Library	337,266.01	
	<u>\$1,962,874.80</u>	
Less Reserve for Depreciation	677,140.22	\$1,285,734.58
Reserve Fund, Securities and Cash		26,830.71
Book Fund, Securities and Cash		<u>18,282.46</u>

\$1,330,847.75

Current Assets:

Cash	\$ 30,467.02	
Accounts Receivable	20,396.05	
Inventories:		
Supply Department	\$ 44,441.66	
Biological Bulletin	20,117.40	64,559.06
Investments:		
Devil's Lane Property	\$ 46,556.99	
Gansett Property	1,749.92	
Stock in General Biological Supply House, Inc.	12,700.00	
Other Investment Stocks	20,095.00	
Retirement Fund	11,517.82	92,619.73
Prepaid Insurance		4,033.08
Items in Suspense		<u>895.41</u>

\$ 212,970.35

Total Assets \$2,510,590.26

Liabilities

Endowment Funds:

Endowment Funds	\$ 948,646.82	
Reserve for Amortization of Bond Premiums..	<u>1,483.22</u>	
		\$ 950,130.04
Minor Funds		<u>16,642.12</u>

\$ 966,772.16

Plant Funds:

Mortgage Notes Payable	\$ 5,000.00	
Donations and Gifts	\$1,172,564.04	
Other Investments in Plant from Gifts and Current Funds	<u>153,283.71</u>	
		<u>\$1,325,847.75</u>

\$1,330,847.75

Current Liabilities and Surplus:

Accounts Payable	\$ 2,754.70	
Items in Suspense	1,799.63	
Reserve for Repairs and Replacements	12,078.12	
Current Surplus	<u>196,337.90</u>	

\$ 212,970.35

Total Liabilities \$2,510,590.26

EXHIBIT B

MARINE BIOLOGICAL LABORATORY INCOME AND EXPENSE,
YEAR ENDED DECEMBER 31, 1945

	Total		Net	
	Expense	Income	Expense	Income
Income:				
General Endowment Fund		\$ 32,214.07		\$ 32,214.07
Library Fund		9,479.18		9,479.18
Donations		755.00		755.00
Instruction	\$ 9,554.39	7,220.00	\$ 2,334.39	
Research	4,550.59	17,434.24		12,883.65
Evening Lectures	86.35		86.35	
Biological Bulletin and Membership Dues.	6,393.65	8,775.63		2,381.98
Supply Department	39,255.03	47,812.56		8,557.53
Mess	24,146.52	20,750.36	3,396.16	
Dormitories	27,443.23	14,547.91	12,895.32	
(Interest and Depreciation charged to above 3 Departments)	(25,574.03)			25,574.03
Dividends, General Biological Supply House, Inc.		14,732.00		14,732.00
Dividends, Other Investment Stocks		725.00		725.00
Rents:				
Bar Neck Property	767.65	6,000.00		5,232.35
Janitor House	30.89	360.00		329.11
Danchakoff Cottages	240.86	275.00		34.14
Sale of Library Duplicates, Micro Film, etc.		344.74		344.74
Microscope and Apparatus Rental		1,372.54		1,372.54
Sundry Income		20.00		20.00
Maintenance of Plant:				
Buildings and Grounds	23,642.27		23,642.27	
Apparatus Department	4,911.52		4,911.52	
Chemical Department	2,265.30		2,265.30	
Library Expense	6,487.95		6,487.95	
Workmen's Compensation Insurance	526.63		526.63	
Truck Expense	238.60		238.60	
Bay Shore Property	92.78		92.78	
Great Cedar Swamp	21.00		21.00	
General Expenses:				
Administration Expense	15,168.99		15,168.99	
Endowment Fund Trustee and Safe-Keep- ing	1,028.45		1,028.45	
Bad Debts	375.97		375.97	
Special Repairs on account of 1944 Hurri- cane Damage	4,297.24		4,297.24	
Interest	125.00		125.00	
Reserve for Depreciation	26,968.12		26,968.12	
	\$173,044.95	\$182,818.23	\$104,862.04	\$114,635.32
Excess of Income over Expense carried to Current Surplus	9,773.28		9,773.28	
	\$182,818.23		\$114,635.32	

EXHIBIT C

MARINE BIOLOGICAL LABORATORY, CURRENT SURPLUS ACCOUNT,
YEAR ENDED DECEMBER 31, 1945

Balance January 1, 1945				\$184,059.96
Add:				
Excess of Income over Expense for Year as shown in Exhibit B..	\$ 9,773.28			
Gain on Gansett Lots Sold	464.18			
Bad Debts Recovered	82.23			
Mortgage Payable, Transferred to Plant Funds	5,000.00			
Reserve for Depreciation Charged to Plant Funds	26,968.12	42,287.81		
				\$226,347.77
Deduct:				
Payments from Current Funds during Year for Plant Assets as shown in Schedule IV:				
Buildings	\$ 7,402.65			
Equipment	4,462.50			
Library	7,500.43			
	\$19,365.58			
Less Received for Plant Assets Sold	5,600.00			
				\$13,765.58
Pensions Paid	\$ 3,460.00			
Loss on Retirement Fund Securities	847.32			
	\$ 4,307.32			
Less Retirement Fund Income	311.79			
				\$ 3,995.53
Transfers to Reserve Fund:				
Portion of Dividends from General Biological Sup- ply House, Inc.	\$ 2,500.00			
Dividends from Crane Co.	625.00			
Income from Operation and Sale of Property 445- 51 W. 23rd and 450-2 W. 24th Sts., N. Y. C.	8,947.72			
Gansett Property Profits, 1944	176.04	12,248.76	30,009.87	
Balance, December 31, 1945				\$196,337.90

Respectfully submitted,

DONALD M. BRODIE,
Treasurer

V. REPORT OF THE LIBRARIAN

The sum \$12,262.54 appropriated to the library in 1945 was expended as follows: books, \$469.99; serials, \$2,625.79; binding, \$577.60; express, \$43.22; supplies, \$147.84; salaries, \$7,262.54 (\$1,150 of this sum was contributed by the Woods Hole Oceanographic Institution); back sets, \$1,104.98; insurance, \$45.00;

sundries, \$5.00; total, \$12,281.96. The cash receipts of the library totalled \$344.74: for microfilms, \$220.34 (\$62.17 expenses paid by the library and accounted above under "supplies"); sale of duplicates, \$122.74; sale of the "Serial List," Biological Bulletin supplement number, \$1.66. This sum, \$344.74, reverts to the laboratory and does not include rent payments for library readers which are collected by the main office. There were 49 library readers accommodated in the library during the summer of 1945.

Of the Carnegie of New York Fund, \$126.35 was expended for the completion of one journal and the partial completion of another.

The sum appropriated by the Woods Hole Oceanographic Institution in 1945 for purchases was \$800. A balance of \$949.39 remaining from 1944 made an available total of \$1,749.39. Of this sum \$947.12 was expended, leaving a balance of \$802.27 towards future purchases. In addition to the above, the Woods Hole Oceanographic Institution contributed \$1,150 (see above under salaries).

During 1945 the library received 902 current journals: 279 (8 new) by subscription to the Marine Biological Laboratory; 30 (7 new) to the Woods Hole Oceanographic Institution; exchanges 352 (6 new; 145 reinstated foreign) and 58 (35 foreign reinstated) with the Woods Hole Oceanographic Institution publications; 177 as gifts to the former and 6 to the latter. The library acquired 206 books: 77 by purchase of the Marine Biological Laboratory; 44 by purchase of the Woods Hole Oceanographic Institution; 10 gifts by the authors; 46 gifts by the publishers; 20 by miscellaneous donors and 9 from Miss Jane Strong. There were 22 back sets of serial publications completed: 14 purchased by the Marine Biological Laboratory (one with the Carnegie Fund); 2 by the Woods Hole Oceanographic Institution; 5 by exchange of the "Biological Bulletin" and one by exchange of duplicate material. Partially completed sets were 54: purchased by the Marine Biology Laboratory, 27 (1 by the Carnegie Fund); purchased by the Woods Hole Oceanographic Institution, 2; by exchange with the "Biological Bulletin," 2; by gift and exchange of duplicate material, 23.

The reprint additions to the library were 4,620; current of 1944, 604; current of 1945, 64; and of previous dates, 3,952. A total of 6,390, 2,130 not duplicates of our holdings, were presented to the library; 4,295 by Mrs. Meigs; 67 by Dr. H. G. Cassidy; 627 by the University of Utah; 26 by Dr. B. M. Davis; and 1,375 by Dr. L. C. Wyman. The large collection of Dr. Garrey's reprints presented last year have not as yet been counted nor started on the way toward cataloguing.

At the end of the year 1945 the library contained 53,990 bound volumes and 137,674 reprints.

Readers of the library report will be glad to note the number of foreign exchanges that have already been reinstated during 1945, both for the "Biological Bulletin" and for the Woods Hole Oceanographic Institution publications: 145 for the former and 35 for the latter. Next year's report will probably show the pre-war number reinstated save only for Germany and perhaps for Russia since we get very poor response from that country. Nor have we heard anything in regard to the German journals on order with Otto Harrassowitz which are apparently stalled if not destroyed in Leipzig. The library committee that is working with the State Department to get these released has nothing so far to report to this library.

VI. REPORT OF THE DIRECTOR

TO THE TRUSTEES OF THE MARINE BIOLOGICAL LABORATORY:

Gentlemen:

I herewith submit a report on the fifty-eighth session of the Marine Biological Laboratory.

1. Attendance

Since 1943 our total attendance has increased from the low point reached that year. In 1944 it was 53 per cent of the pre-war average of 490; in 1945 it was 63 per cent. This increase is found among the independent investigators and the students; the beginning investigators and research assistants, who, as I explained in the last report, belong in one group, are still sparsely represented. In 1945 there were only 36, whereas the pre-war average was 130. The advance registration for 1946 shows an encouraging increase in this group. Many of the applicants are veterans who are taking advantage of Government funds provided under the G. I. Bill of Rights.

2. Building Repairs

One of the inevitable effects of the war has been the deterioration of our buildings. Lack of materials and labor has up until now prevented all but the most essential repairs from being made. Fortunately, we are now able to begin to put our house in order, in spite of the shortage of some critical materials. To determine what work should be done, a Committee on Special Repairs, under the able leadership of Mr. C. L. Claff, conducted a thorough survey of all of the buildings and made detailed recommendations. The report, a model of completeness as drawn up by Mr. Claff, calls for the ultimate expenditure of approximately \$145,000 for present repairs and future desirable improvements not only in the buildings but also in equipment for the Apparatus Department and the Supply Department.

The Executive Committee voted to expend the entire Reserve Fund, amounting to \$25,000, and all but a minimum of the current cash on hand for making the most urgently needed repairs at once. It also laid plans for securing outside funds with which to complete the changes called for in the report, and to purchase apparatus. In addition, funds for a new building and for additional endowment are to be sought.

Many of the essential repairs have already been made. The Mess kitchen, never properly restored after the Navy occupation, is now in good condition, and improvements in the dining room have been made. The Botany Building, unused for several seasons, has been put to rights with new plumbing, wall tables, shelves, and other fixtures. Replacements have been made in the Supply Department and Rockefeller Building; hot water systems are installed in the residences heretofore not so provided; and much painting has been done. This work was accomplished in large measure by our permanent staff, under the direction of Mr. MacNaught. All the men worked faithfully and energetically, and have completed the assigned tasks in a most satisfactory manner. It is hoped that waterproofing of the Crane and Brick Buildings may be completed before the 1946 season begins. As soon as this has been satisfactorily finished, those Laboratory rooms which have been damaged by water can be made presentable.

3. *The Housing Problem*

An unexpected outcome of war-time activities is the housing shortage. Before the war our residences and the houses in the village could accommodate 450 to 500 persons during the course of a summer. But when the Oceanographic Institution embarked on extensive defense projects, the number of its workers increased from comparatively few to upwards of 250, most of whom are year round residents. They now occupy most of the available houses in the village; some are forced to live as far away as North Falmouth and Hyannis. As a result of this crowding we shall be unable, in the summer of 1946, to take care of more than 375 investigators and students—that is, about 100 less than our pre-war average. Indeed, we can accommodate this number only because the authorities of the U. S. Fish and Wild Life Commission have granted us the use of a part of the Fisheries residence. For their cooperation in this, and in many other ways, the Laboratory is grateful.

When it is possible once more to build houses, some of this pressure for living space will be relieved. To encourage investigators to have homes here in Woods Hole, the Laboratory has opened up the Devil's Lane tract, situated a mile and more from the center of the village, between the State Road to Falmouth and the railroad. About 100 lots will presently be available.

In the meantime, the number of applicants for research space will undoubtedly increase, and we shall be unable to find places for all qualified investigators who wish to come. The Administration thus faces the unwelcome prospect of having to choose between applicants. The Executive Committee has ruled that investigators, instructors, and students should have preference over Library readers in the residences and at the Mess. But some further method of selection must be followed until the housing shortage is relieved.

4. *Financial Problems*

The report of the Treasurer shows that our financial condition is sound; that is, we are free from debt, and have about \$57,000 in the Reserve and Current Cash accounts. But most of this has already been earmarked to pay for the most necessary repairs, and for foreign journals not yet delivered. We shall still need a larger amount for other needed repairs and replacements. When these have been made we can say that our regular income from all sources is sufficient to maintain the Laboratory on its present basis. But in order to expand our research facilities we must have additional funds. It is estimated that \$30,000 each year should be spent for this purpose.

5. *Gifts*

Mr. Allen R. Memhard has provided a fund of \$1,000, the income of which may be awarded to a qualified student who has completed the Embryology course.

Mrs. Adele K. Stricker has presented to the Laboratory the sum of \$50 in memory of her son, Capt. George J. Stricker, who worked here during the summers of 1933 and 1934.

Dr. A. C. Redfield contributed \$100 for a hedge and trees to be planted to the east of the Stone Building.

Donations for current purposes received during the year were as follows: Mrs. E. B. Meigs, \$25.00; Dr. William D. Curtis, \$100.00; M. B. L. Associates, \$630.00.

6. Deaths

This year we have sustained irreparable losses by death; Dr. T. H. Morgan, Trustee since 1897, whose scientific achievements and devotion to this Laboratory from its earliest days contributed greatly to its growth in usefulness and influence, and Dr. C. E. McClung, elected Trustee in 1913, active in all Laboratory affairs, especially in the building up of our great Library.

7. Election of Trustees

At the meeting of the Corporation, held August 14, 1945, the following were elected Trustees Emeriti: Dr. F. P. Knowlton, elected Trustee in 1922; Dr. R. S. Lillie, elected Trustee in 1921.

The following were elected Trustees: Dr. P. B. Armstrong, Professor of Anatomy, College of Medicine, Syracuse University; Dr. A. K. Parpart, Associate Professor of Biology, Princeton University.

8. Publications

The Executive Committee voted to print in this Report a list of papers, based wholly or in part on work done at this Laboratory, and published during the years 1941-1945. A similar list, which appeared in the Annual Report of 1908, covered the years from the beginning of the Laboratory in 1888 to 1907. It is hoped that eventually a complete compilation of titles to include the intervening years may be made.

Appended as parts of this Report are:

1. Publications from this Laboratory during the years 1941-1945.
2. The Staff.
3. Investigators and Students.
4. Tabular View of Attendance, 1941-1945.
5. Subscribing and Cooperating Institutions.
6. Evening Lectures.
7. Shorter Scientific Papers.
8. Members of the Corporation.

Respectfully submitted,
CHARLES PACKARD,
Director

1. PUBLICATIONS FROM THE MARINE BIOLOGICAL LABORATORY, 1941-1945

Note: An asterisk before a title indicates that the work was done only in part at this Laboratory.

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- S. O. MAST, Professor of Zoology, Emeritus, Johns Hopkins University.
- A. P. MATHEWS, Professor of Biochemistry, Emeritus, University of Cincinnati.
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JANE M. OPPENHEIMER, Assistant Professor in Biology, Bryn Mawr College.

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IV. LABORATORY ASSISTANTS

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ARTHUR C. GIESE, Associate Professor of Biology, Stanford University (absent in 1945).
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 COLWIN, LAURA HUNTER, Instructor, Pennsylvania College for Women.
 FRIEDEMANN, ULRICH H., Head of Department of Bacteriology, Brooklyn Jewish Hospital.
 FRISCH, JOHN A., Professor of Biology, Canisius College.
 GATES, R. RUGGLES, Professor Emeritus, University of London.
 GUREWICH, VLADIMIR, Assistant Visiting Physician, Bellevue Hospital.
 KABAT, ELVIN A., Research Associate in Biochemistry, College of Physicians and Surgeons.
 KAYLOR, CORNELIUS T., Assistant Professor of Anatomy, Syracuse University.
 KELLER, RUDOLPH, Researcher, Robinson Foundation, New York.
 KRASNOW, FRANCES, Head of Department of Research, Guggenheim Dental Foundation.
 LANGE, MATHILDE M., Professor of Zoology, Head of Department of Biology, Wheaton College.
 LOEWI, OTTO, Research Professor of Pharmacology, New York University, College of Medicine.
 MARINELLI, LEONIDAS, Physicist, Memorial Hospital.
 MAJOR, JAMES W., Professor of Biology, Union College.
 MAYER, MANFRED M., Scientific Staff, War Research Division, Columbia University.
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 MEYERHOF, DR. OTTO, Research Professor of Biochemistry, University of Pennsylvania.
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 RYAN, FRANCIS J., Assistant Professor, Columbia University.
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 STRAUSS, WILLIAM L., JR., Associate Professor of Anatomy, Johns Hopkins University.
 VONDACH, HERMAN, Assistant Professor of Physiology, Georgetown Medical School.
 WALLACH, JACQUES B., Long Island College of Medicine.
 ZORZOLI, ANITA, Assistant Instructor, New York University.

Students, 1945

BOTANY

BARRACLOUGH, MARY EDITH, Student, Smith College.
 DIETZ, ALMA, Assistant in Biology, American International College.
 GARDNER, ELIZABETH B., Radcliffe College.
 MOUL, EDWIN THEODORE, Botany Assistant, University of Pennsylvania.
 SMITH, MATTIE LOU, Student, Radcliffe College.

EMBRYOLOGY

BEACH, JANET, Student, University of Connecticut.
 BERNIER, GERMAINE, University of Montreal, Quebec, Canada.
 BERRY, BETH SINCLAIR, Student, Rockford College, Illinois.
 CARTER, MARJORIE ESTELLE, Teacher, Georgia State Women's College.
 CHIRICO, ANNA MARIE, Student, Seton Hill College.
 CLARK, CARL CYRUS, Student, Amherst College.
 COPINGER, ANNE STEVENS, Goucher College.
 EERLICH, MIRIAM, Knox College.
 IZZO, MARY JANE, University of Rochester.
 KELL, AMY, University of Illinois.
 LEVIN, ILANE B., Goucher College.

LODICO, DOROTHY GERALDINI, University of Rochester.
LOVELACE, LOLLIE ROBERTA, Teaching Fellow, University of North Carolina.
MARKER, MURIEL JOSEPHINE, Student, Colby College.
MEYGER, LISELOTTE, Student, Bryn Mawr College.
MILLER, HELMA C., Graduate Assistant, Johns Hopkins University.
PERKINS, BARBARA BURNHAM, University of Connecticut.
RAYMOND, BARBARA, Student, Swarthmore College.
RICE, MARY ESTHER, Assistant in Biology Laboratory, Drew University.
ROBERTS, ELIZABETH S., Assistant in Biology, Wilson College.
RUDERMAN, CLAIRE, Teaching Assistant, University of Rochester.
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UPHOFF, DELTA E., University of Rochester.

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COOK, JOHN ALFRED, George Washington University.
FERGUSON, ALICE HOWARD, Graduate Assistant, Louisiana State University.
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FOSTER, ELIZABETH JANE, Student, University of Illinois.
GOLDSMITH, YVETTE, Perth Amboy, New Jersey.
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BATES, MARY FLORENCE, Student, Vassar College.
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BENJAMIN, MRS. REZSIN C., Undergraduate Student, University of Rochester.
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BERNIER, GERMAINE, Instructor, University of Montreal.
BEZILLA, HELEN, Student, Seton Hill College.
BRADIN, JOHN L., Northwestern University.
CALVERT, JULIE NEIL, Student, Wilson College.
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CLARK, CARL CYRUS, Amherst College.
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DAILEY, DOROTHY HELEN, Depauw University.
DAWSON, MARY JEAN, Student, Mt. Holyoke College.
DEMPSEY, ELLEN, Oberlin College.
DICKASON, MARY ELIZABETH, Student, Smith College.
FARNEHAM, CAROL JEAN, Student, Drury College.
FREITAG, JANET FAITH, Student, University of Connecticut.
GOLDIS, BERNICE RUTH, Graduate Student, University of Pennsylvania.
HANLON, REV. JAMES J., Graduate Student, Fordham University.
HILL, SHIRLEY B., Student, Vassar College.
HINES, EILEEN BARBARA, State University of Iowa.
JONES, DOROTHY B., Student, University of Connecticut.
JOSITA, SISTER M., Student, Fordham University.
JULIER, EDITH VAILLANT, Student, Vassar College.

KREKELER, CARL H., Student, Washington University.
 KUHN, ALICE ROBERTS, Western-Maryland College.
 LOWENS, MARY DOROTHY, Student, Swarthmore College.
 McCLAIN, MARYLOW, Student, Swarthmore College.
 MCGREGOR, ELIZABETH, Instructor, Mount Holyoke College.
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 RAYMOND, BARBARA, Student, Swarthmore College.
 RIGGS, AUSTIN F., Student, Harvard University.
 ROGERS, HENRY CRAMPTON, Deerfield Academy.
 SCHAEFER, GERTRUDE, Undergraduate, Temple University.
 SEAMAN, ARLENE, Zoology Assistant, Cornell University.
 SNIPES, ANNE, Wheaton College.
 STEES, NANCY, Teacher, West Chester State Teachers College.
 SURREARER, THOMAS C., Professor of Biology, Baldwin-Wallace College.
 THORNTON, DOROTHY GOLDEN, Assistant in Zoology Dept., Wellesley College.
 TUPPER, LYLA, Graduate Student, Northwestern University.
 UBER, VIRGINIA M., Student, Pennsylvania College for Women.
 WAX, FLORENCE SIMA, Student, Oberlin College.
 WHYTE, MARJORIE ANN, Assistant, Cornell University.
 WILCOX, BARBARA L., Student, Radcliffe College.
 WILLIAMS, OLWEN, Teacher of Biology and Chemistry, The Putney School.
 WILSON, FAITH EVELYN, Johns Hopkins University.
 WILSON, MARIE ELLEN, Student, Western Maryland College.

4. TABULAR VIEW OF ATTENDANCE

	1941	1942	1943	1944	1945
INVESTIGATORS—Total	337	201	160	193	212
Independent	197	132	89	112	138
Under instruction	59	16	19	11	10
Library readers	31	28	35	50	38
Research assistants	50	25	17	20	26
STUDENTS—Total	131	74	68	75	96
Zoology	55	36	47	37	55
Embryology	37	24	13	23	23
Physiology	24	6	8	10	13
Botany	15	8	—	5	5
TOTAL ATTENDANCE	468	275	228	276	308
Less persons registered as both students and investigators	7	2	6	1	
	461	273	222	275	
INSTITUTIONS REPRESENTED—Total	144	126	116	106	124
By investigators	102	83	70	74	100
By students	72	43	41	41	49
SCHOOLS AND ACADEMIES REPRESENTED					
By investigators	5	2	2	1	2
By students	2	—	1	2	2
FOREIGN INSTITUTIONS REPRESENTED					
By investigators	3	—	2	2	1
By students	1	—	—	3	—

5. SUBSCRIBING AND COOPERATING INSTITUTIONS

1945

Albany Medical College	Oberlin College
Amherst College	Ohio State University
Biological Institute, Philadelphia, Pennsylvania	Pennsylvania College for Women
Bowdoin College	Princeton University
Bryn Mawr College	Radcliffe College
Cathedral College	Rockefeller Institute for Medical Research
The Catholic University of America	St. Joseph College for Women
Columbia University	Smith College
Cornell University	State University of Iowa
Cornell University Medical College	Syracuse University
Duke University	Syracuse University Medical School
Fish and Wild Life Service, U. S. Dept. of the Interior	Temple University
Fordham University	University of Chicago
Goucher College	University of Connecticut
Harvard University	University of Illinois
Harvard University Medical School	University of Maryland Medical School
Industrial and Engineering Chemistry, of the American Chemical Society	University of Michigan
Johns Hopkins University	University of Missouri
Johns Hopkins Medical School	University of Pennsylvania
Lee Foundation	University of Pennsylvania School of Medicine
Eli Lilly & Company	University of Rochester
Long Island University	Vanderbilt University Medical School
Macy Foundation	Vassar College
Massachusetts Institute of Technology	Washington University
McGill University	Wayne University
Miami University	Wellesley College
Mount Holyoke College	Wesleyan University
New York University	Western Maryland College
New York University College of Medicine	Western Reserve University
New York University School of Dentistry	Wheaton College
New York University Washington Square College	Williams College
	Wilson College
	Woods Hole Oceanographic Institution
	Yale University

6. EVENING LECTURES, 1945

Friday, June 29

PROF. P. W. WHITING "The Development of Hymenopteran Genetics."

Friday, July 6

DR. R. R. GATES "Human Heredity in Relation to Animal Genetics."

Friday, July 13

DR. I. FANKUCHEN "X-Ray Diffraction and Protein Structure."

Friday, July 20

PROF. S. C. BROOKS "Our Interrelationships with South American Universities, together with Illustrated Travel Notes."

Friday, July 27

PROF. E. G. BUTLER "Problems of Differentiation and Dedifferentiation in Amputated Urodele Limbs."

Friday, August 3

DR. BOSTWICK H. KETCHUM "The Prevention of Ship Bottom Fouling."

Friday, August 10

DR. DANIEL MERRIMAN "A Study in Pure and Applied Marine Biology. The Life History and Economic Importance of the Ocean Pout."

Friday, August 17

DR. DETLEV W. BRONK "Biological Research During the War and Postwar Periods."

Friday, August 24

DR. F. L. HISAW "Endocrines and the Evolution of Viviparity among the Vertebrates."

Monday, August 27

GEORGE G. LOWER "Local Invertebrates."

Wednesday, August 29

DR. PAUL S. GALTISOFF "Impressions of a Biologist at the San Francisco Conference."

Thursday, August 30

MAJOR A. H. NEUFELD "Medical Research Organization in the Canadian Army."

Thursday, August 30

CAPT. W. R. DURYEE "Medical Military Training."

7. SHORTER SCIENTIFIC PAPERS, 1945

Tuesday, July 24

DR. M. M. BROOKS "The Redox Potential of *Penicillium rotatum* Medium under Some Different Conditions of Growth."

DR. WILBUR ROBBIE "The Use of Cyanide in Manometric Experimentation."

DR. SEARS CROWELL "The Displacement of Terns by Gulls at Weepeket Island."

Tuesday, July 31

DR. P. W. WHITING "The Problem of Reversal of Male Haploidy by Selection."

DR. BERTA SCHARRER "Experimental Tumors after Nerve Section in an Insect."

DR. P. S. GALTISOFF "Reactions of Oysters to Free Chlorine."

Tuesday, August 7

DR. T. H. BULLOCK "Organization of Giant Nerve Fibers in certain Polychaetes."

DR. ERNST SCHARRER "The Origin of Neurosecretory Granules from Basophil Substances in the Nerve Cells of Fishes."

DR. C. H. TAFT "The Action of Quinine on the Livers of Tautog and Toadfish."

DR. A. M. SHANES "Evidence of a Metabolic Effect by Potassium in Lowering the Injury Potential of Nerve."

Tuesday, August 14

- DR. R. CHAMBERS "Interrelations between Sperm-Nucleus, Egg-Nucleus and Cytoplasm in *Asterias* Egg."
- DR. KURT G. STERN "Physical-chemical Studies on Chromosomal Nucleoproteins."

Tuesday, August 21

- DR. DOROTHY WRINCH "Hemoglobin and other Native Proteins."
- DR. E. R. WITKUS "Endomitosis in Plants."
- DR. C. A. BERGER "Recent Cytological Studies in *Culex*."

Thursday, August 23

- DR. ETHEL B. HARVEY "Development of Granule-free Fractions of *Arbacia* eggs."
- DR. ALEXANDER SANDOW "Studies of the Muscle Twitch by Methods of Electronic Recording."
- DR. C. D. BEERS "The Role of Bacteria in the Excystment of the Ciliate *Didinium*."

Monday, August 27

- DR. ANNA R. WHITING "Differences in Sensitivity, Hatchability Curves and Cytological Effects between Eggs X-rayed in First Meiotic Prophase and Metaphase."
- DR. W. W. WAINIO "Aerobic Oxidation of Simple sugars by Mammalian Liver."
- DR. DUGALD E. S. BROWN "The Role of Myosin and Myosin Triphosphatase in *Vitro* and in Muscle."

Tuesday, August 28

- DR. LLOYD M. BERTHOLF "Accelerating Metamorphosis in the Tunicate *Styela*."
- DR. ALFRED FROELICH "The Influence of Drugs on Heat-narcosis."
- DR. W. MALCOLM REID "*In Vivo* and *in Vitro* Glycogen Utilization in the Avial Nematode *Ascaridia Galli*."

8. MEMBERS OF THE CORPORATION, 1945

1. LIFE MEMBERS

- ALLIS, MR. E. P., JR., Palais Carnoles, Menton, France.
- BECKWITH, DR. CORA J., Vassar College, Poughkeepsie, New York.
- BILLINGS, MR. R. C., 66 Franklin Street, Boston, Massachusetts.
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- COLE, DR. LEON J., College of Agriculture, Madison, Wisconsin.
- CONKLIN, PROF. EDWIN G., Princeton University, Princeton, New Jersey.
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- JACKSON, MISS M. C., 88 Marlboro Street, Boston, Massachusetts.
- KING, MR. CHAS. A.

KINGSBURY, PROF. B. F., Cornell University, Ithaca, New York.
LEWIS, PROF. W. H., Johns Hopkins University, Baltimore, Maryland.
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MOORE, DR. GEORGE T., Missouri Botanical Gardens, St. Louis, Missouri.
MOORE, DR. J. PERCY, University of Pennsylvania, Philadelphia, Pa.
MORGAN, MRS. T. H., Pasadena, California.
MORGAN, PROF. T. H., Director of Biological Laboratory, California Institute of Technology, Pasadena, California.
NOYES, MISS EVA J.
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SHEDD, MR. E. A.
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2 REGULAR MEMBERS

ADAMS, DR. A. ELIZABETH, Mount Holyoke College, South Hadley, Massachusetts.
ADDISON, DR. W. H. F., University of Pennsylvania Medical School, Philadelphia, Pennsylvania.
ADOLPH, DR. EDWARD F., University of Rochester Medical School, Rochester, New York.
ALBAUM, DR. HARRY G., Biology Dept., Brooklyn College, Brooklyn, N. Y.
ALBERT, DR. ALEXANDER, 383 Harvard Street, Cambridge, Mass.
ALLEE, DR. W. C., The University of Chicago, Chicago, Illinois.
AMBERSON, DR. WILLIAM R., Department of Physiology, University of Maryland, School of Medicine, Lombard and Greene Streets, Baltimore, Maryland.
ANDERSON, DR. RUBERT S., University of Maryland School of Medicine, Department of Physiology, Baltimore, Maryland.
ANDERSON, DR. T. F., University of Pennsylvania, Philadelphia, Pennsylvania.
ARMSTRONG, DR. PHILIP B., College of Medicine, Syracuse University, Syracuse, New York.
AUSTIN, DR. MARY L., Wellesley College, Wellesley, Massachusetts.
BAITSELL, DR. GEORGE A., Yale University, New Haven, Connecticut.
BAKER, DR. H. B., Zoological Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania.
BALLARD, DR. WILLIAM W., Dartmouth College, Hanover, New Hampshire.
BALLENTINE, DR. ROBERT, Columbia University, Department of Zoology, New York City, New York.
BALL, DR. ERIC G., Department of Biological Chemistry, Harvard University Medical School, Boston, Massachusetts.
BARD, PROF. PHILIP, Johns Hopkins Medical School, Baltimore, Maryland.
BARRON, DR. E. S. GUZMAN, Department of Medicine, The University of Chicago, Chicago, Illinois.
BARTH, DR. L. G., Department of Zoology, Columbia University, New York City, New York.

- BARTLETT, DR. JAMES H., Department of Physics, University of Illinois, Urbana, Illinois.
- BEADLE, DR. G. W., School of Biological Sciences, Stanford University, California.
- BEAMS, DR. HAROLD W., Department of Zoology, State University of Iowa, Iowa City, Iowa.
- BECK, DR. L. V., Hahnemann Medical College, Philadelphia, Pennsylvania.
- BEHRE, DR. ELINOR H., Louisiana State University, Baton Rouge, Louisiana.
- BERTHOLF, DR. LLOYD M., Western Maryland College, Westminster, Maryland.
- BIGELOW, DR. H. B., Museum of Comparative Zoology, Cambridge, Massachusetts.
- BIGELOW, PROF. R. P., Massachusetts Institute of Technology, Cambridge, Massachusetts.
- BINFORD, PROF. RAYMOND, Guilford College, North Carolina.
- BISSONNETTE, DR. T. HUME, Trinity College, Hartford, Connecticut.
- BLANCHARD, PROF. K. C., Johns Hopkins Medical School, Baltimore, Maryland.
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- BROOKS, DR. MATILDA M., University of California, Department of Zoology, Berkeley, California.
- BROOKS, DR. S. C., University of California, Berkeley, California.
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- BUCK, DR. JOHN B., Industrial Hygiene Research Lab., National Institute of Health, Bethesda, Maryland.
- BUCKINGHAM, MISS EDITH N., Sudbury, Massachusetts.
- BUDINGTON, PROF. R. A., Winter Park, Florida.
- BULLINGTON, DR. W. E., Randolph-Macon College, Ashland, Virginia.
- BULLOCK, DR. T. L., University of Missouri, Columbia, Missouri.
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COSTELLO, DR. HELEN MILLER, Department of Zoology, University of North Carolina, Chapel Hill, North Carolina.
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CRANE, JOHN O., Woods Hole, Massachusetts.
CRANE, MRS. W. MURRAY, Woods Hole, Massachusetts.
CROASDALE, HANNAH T., Dartmouth College, Hanover, New Hampshire.
CROUSE, DR. HELEN V., University of Pennsylvania, Philadelphia, Pennsylvania.
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- GREGORY, DR. LOUISE H., Barnard College, Columbia University, New York City, New York.
- GUDERNATSCH, DR. J. FREDERICK, New York University, 100 Washington Square, New York City, New York.
- GUTHRIE, DR. MARY J., University of Missouri, Columbia, Missouri.
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THE INFLUENCE OF TEXTURE AND COMPOSITION OF SURFACE ON THE ATTACHMENT OF SEDENTARY MARINE ORGANISMS *

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Marine installations of various kinds necessitate exposure of construction materials under sea water. Data dealing with the amount of fouling accumulated by such materials are not abundant. Information which might be of aid to the scientist seeking the most favorable material upon which to collect sedentary organisms for study is also scanty. The present study was undertaken to determine the effect

TABLE I

Effect of surface texture of glass on attachment of sedentary organisms. (Numbers of individuals on each surface of 80 square inches of plate)

Surface number	Plain 0	Sand- blasted 1	Factrolite 2	Prestlite 3	Ribbed 4	Pentecor 5
<i>Series No. 1, Tahiti Beach¹</i>						
39 days (8/22/42-9/30/42)						
<i>Hydroides</i> sp.	143	265	152	506	349	197
<i>Spirorbis</i> sp.	85	188	122	90	163	110
Barnacles	1,948	1,072	1,162	975	1,674	2,140
Total	2,176	1,525	1,436	1,571	2,186	2,447
Average pop.	725.3	508.3	478.7	523.7	728.7	815.7
Average/square inch	9.1	6.4	6.0	6.5	9.1	10.2
<i>Series No. 2, Miami Beach²</i>						
17 days (8/22/42-9/8/42)						
Wet weight (grams)	51.0	45.5	50.0	41.0	50.0	41.0
Dry weight (grams)	8.5	6.4	7.9	5.5	7.5	8.8
Barnacles	308	227	268	213	263	331
<i>Series No. 3, Miami Beach²</i>						
30 days (9/15/42-10/15/42)						
Wet weight (grams)	164.5	174.0	149.0	150.0	126.0	155.5
Dry weight (grams)	51.5	50.0	30.0	24.0	24.0	33.0
Barnacles	642	515	554	778	798	977

¹ Subtropical testing service.

² Beach boat slips.

* The observations described here were made while the authors were engaged by the Woods Hole Oceanographic Institution in an investigation of fouling, under contract with the Bureau of Ships, Navy Department, which has given permission for their publication. The opinions presented here are those of the authors and do not necessarily reflect the official opinion of the Navy Department or the naval service at large. Contribution No. 349 from the Woods Hole Oceanographic Institution.

of surface irregularities and of substrate composition on the establishment of sessile populations. The experiments were conducted in Biscayne Bay at Miami, Florida, where subtropical conditions favor the attachment of fouling organisms throughout the year.

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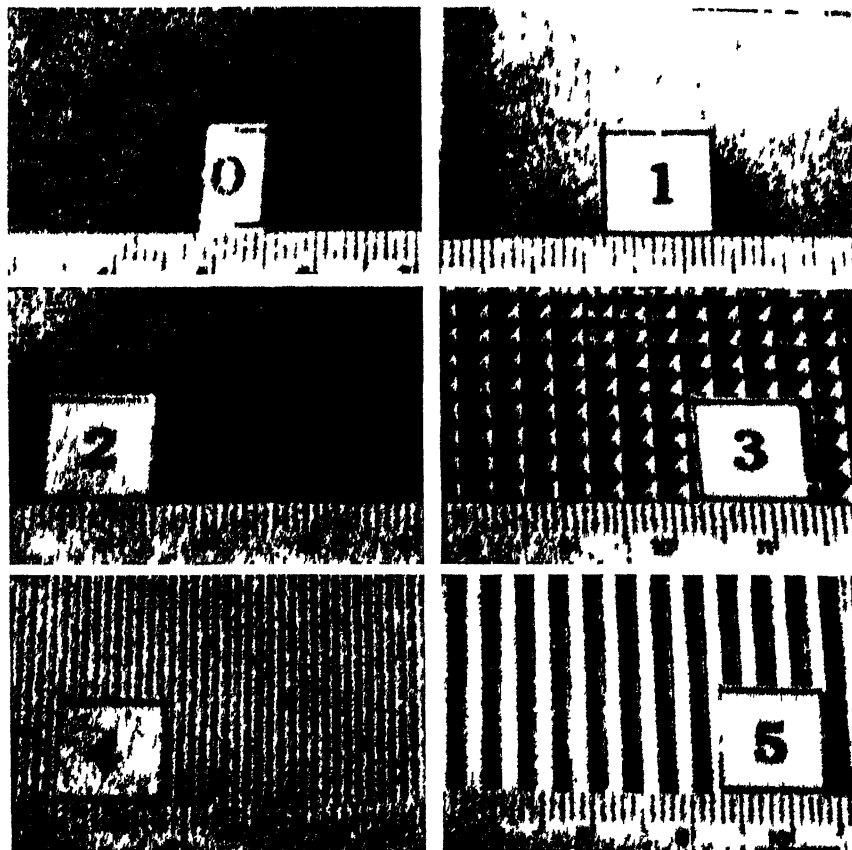


FIGURE 1. Glass surfaces used in testing the relation of surface irregularities to fouling 0. Plain 1 Sandblasted 2 Factrolite. 3. Prestlite. 4 Ribbed 5 Pentecor.

EFFECT OF SURFACE IRREGULARITY

Commercial glasses, manufactured by the Pittsburgh Glass Company, with various surface irregularities were used for this study. Six 8 × 10 inch glass plates were assembled, irregular surface down, in a rack suitable for floating on the surface of the water. The floats were constructed in such a way that sea water could move freely on both sides of the exposed surface. The backs of the panels which were all relatively smooth were placed upward. The fouling on the back surfaces was

not recorded. The surface irregularities of the panels are shown in Figure 1 and may be described as follows:

Surface Number:

0. *Plain* Smooth glass, polished.
1. *Sandblasted* Glass sandblasted on lower side.
2. *Factrolite* Surface consisted of pyramidal depressions of which there were about 144 per square centimeter.
3. *Preclite* Approximately nine pyramidal depressions per square centimeter.
4. *Ribbed* Surface of V-shaped grooves, nine grooves per centimeter of width.
5. *Pentecor* Approximately three V-shaped grooves per centimeter of width.

Results obtained from three series of experiments in which the glass surfaces were exposed are shown in Table I.

The Sessile populations which grew on the glass plates were composed primarily of barnacles and tubeworms, with irregular, perhaps seasonal, appearances of tunicates and *Anomia* sp. Barnacles (*B. improvisus* and *B. amphitrite niveus* in order of relative abundance) were numerous in both locations, but those at Tahiti Beach were always very small compared to those at Miami Beach. Many more barnacles attached to the lower (shaded) surfaces of the panels than to the upper surfaces where light was more abundant. This is in agreement with the experience of Pomerat and Reiner (1942), who report that larger numbers of barnacles accumulate on dark surfaces than on light surfaces. The shaded undersides of the glass panels, being darker than the upper sides, appear to attract more cyprids and hence show a greater barnacle accumulation.

The various surface textures of glass had little influence on the number of attached organisms. In these experiments barnacles were consistently slightly more numerous on Pentecor than on smooth glass. This behavior was confirmed in the experiment reported in the following section although conditions of exposure were not exactly parallel. In the first experiment the glass panels were floated at the surface in a shaded location, while in the second they hung vertically below low tide

TABLE II

Influence of substrate on fouling, sixty days' exposure at the beach boat slips, September 25, 1942–November 25, 1942

Substrate	Weight of fouling on panel area of 264 sq. in., that of wood panels employed	
	Wet weight grams	Dry wt. grams
1. Dade County pine	675.1	346.5
2. Gum	1127.6	531.4
3. Magnolia	1165.4	446.4
4. White pine	968.7	446.8
5. Cypress	954.8	392.0
6. Tile	† 980.1*	487.3
7. Cement	1033.0*	534.1
8. Glass	386.1	167.3

* Corrected to an area, 264 sq. in., equal to that of the wood panels.

under sun exposure. Counts of tubeworms were made on only one set of exposures. *Hydroids* sp. was most abundant on Prestlite and *Spirorbis* sp. was most abundant on sandblasted glass.

COMPOSITION OF THE SURFACE

Unpainted panels of wood of five species, clay roofing tiles, cement roofing plates, and a glass panel were exposed for 60 days at the Beach Boat Slips in Miami Beach.

TABLE III

Effect of substrate on fouling, exposures of three months at South Dock, Belle Isle, Miami Beach, Florida, January 9, 1943-April 9, 1943, all materials applied to, or mounted on, wood unless otherwise noted

Composition of surfaces	Wet weight* (grams)	Dry weight* (grams)	Number* of barnacles	Notes
<i>Plastics</i>				
1. Celluloid	3.8	2.2	11	Thin coat of algae.
2. Plasticel	24.3	12.2	124	Barnacles' bases easily removed.
3. Lucite	5.6	1.7	41	
4. Formica	6.9	3.2	11	
5. Isobutyl Methacrylate	15.4	7.2	70	Film applied to glass panel. Plastic peels intact with barnacles.
<i>Glass</i>				
6. Prestlite	57.0	25.2	176	Some barnacles 12 mm. across.
7. Pentecor	46.0	25.0	148	Some barnacles 12 mm. across.
8. Sandblasted	23.6	7.0	46	6 calcareous tubeworms; tunicates.
9. Smooth	4.5	1.7	16	Green slime may have caused fish to remove young barnacles.
<i>Paints and ingredients**</i>				
<i>Coatings applied to steel panels</i>				
10. Ester gum vehicle	36.3	8.1	58	Tunicates and bryozoa.
11. Rosin vehicle	2.7	0.4	0	Fish spawn both sides.
12. Anticorrosive paint 42-A	2.7	0.5	9	Barnacles very small.
13. Vehicle of 15RC	6.1	3.3	43	
14. Antifouling paint 7C	0.0	0.0	0	Some slime film.
15. Antifouling paint 8C	0.6	0.3	14	Small barnacles close to edge.
<i>Coatings Applied to Wood</i>				
16. Ceraloid	57.6	38.5	183	
17. Paraffin	11.3	6.1	59	Lomnoria active in breaking paraffin.
18. Asphaltum	121.4	34.3	768	Barnacles only.
19. Asphaltum varnish	67.8	13.8	256	Some bryozoa.
20. Spar varnish	45.1	7.0	304	
21. Navy grey	41.6	5.6	150	Algae.
22. Anti-corrosive 42-A	48.2	10.7	156	

* * Corrected to an area of 144 square inches.

** Anticorrosive 42A is a standard Navy formula. Vehicle of 15RC is the non-pigmented portion of a standard Navy antifouling paint. Antifouling paints 7C and 8C are experimental modifications of a standard Navy antifouling paint of the cold plastic type in which the toxic pigment is reduced to 50 and 60 percent of the normal value.

TABLE III—*Continued*

Composition of surfaces	Wet weight* (grams)	Dry weight* (grams)	Number* of barnacles	Notes
<i>Woods</i>				
23. Dade County pine (soaked 60 days)	395.2	120.7	748	Bryozoa.
24. Gum (soaked 60 days)	452.1	133.4	686	
25. Dade County pine (unsoaked)	144.3	27.3	125	Hydrozoa, bryozoa.
26. Gum (unsoaked)	249.8	43.5	222	
27. Soft pine	57.6	11.5	184	
28. Teak	143.8	88.7	306	Large barnacles.
29. Maderia	173.7	84.2	358	Many fish eggs.
30. Greenheart	77.0	40.8	342	
31. Balsa	2.9	1.6	5	Wood very soft.
<i>Metals</i>				
32. Steel	224.4	42.8	88	
33. Galvanized iron	2.6	0.7	6	Barnacles easily removed.
34. Zinc	1.0	0.2	0	Active corrosion.
35. Lead	30.6	50.9	396	Large barnacles.
36. Monel	1.6	0.5	6	Many fish eggs.
37. Nickel	43.2	10.7	126	
38. Galvanized iron pipe	4.7	3.0	27	Barnacle on rusted threads and damaged edges.
<i>Miscellaneous</i>				
39. Linoleum	79.7	23.0	193	
40. Deck canvas no. 10	5.1	2.3	7	Sagging-algae eaten by fish.
41. Sole leather	32.4	12.4	66	
42. Masonite, heat tempered	138.6	31.8	594	Brown tunicates.
43. Asbestos	284.2	65.9	980	Bryozoa, <i>Anomia</i> , hydrozoa, calcareous tubeworm.

All panels were suspended in a vertical position approximately two feet beneath the mean low water mark. The site was well shaded by a protecting roof. The results obtained are presented in Table II.

The weights of the populations (barnacles, tubeworms, tunicates, bryozoa, and algae) which accumulated on the woods, tile, and cement were of the same order of magnitude, though variations as great as 59 per cent were observed. The weight of organisms accumulated on glass was approximately 30 per cent of that collected from other substrate materials.

A much larger number of materials were tested in a second experiment, the results of which are given in Table III. Exposure was made for three months at South Dock, Belle Isle, in Miami Beach, where conditions of bright sunlight, active current movement, and moderate fouling incidence were found. Growth on the panels consisted primarily of barnacles (*B. improvisus* and *B. amphitrite nivicus*) with occasional tufts of hydrozoa and patches of colonial tunicates. A blanket of algae having very short filaments grew on panels of light color or shaded background. Large sets of fish eggs were found on the rosin vehicle and Monel. Borings of *Limnoria* sp. were everywhere evident in unprotected wood.

The substrates accumulating heaviest populations were asbestos, asphaltum, Dade County pine (pre-soaked 60 days), gum wood (pre-soaked 60 days) and Masonite. Asbestos shingle, commonly used as clapboarding, yielded the richest harvest as measured by the number of barnacles. A comparison of asbestos and Masonite, two of the best collectors, is shown in Figure 2. The asphaltum used was of the type employed as aquarium cement. It accumulated barnacles only.

Panels of gum and Dade County pine, which had been exposed for 60 days in the earlier test reported in Table II, were included for comparison with unsoaked specimens of these woods. The unsoaked woods developed much less fouling.

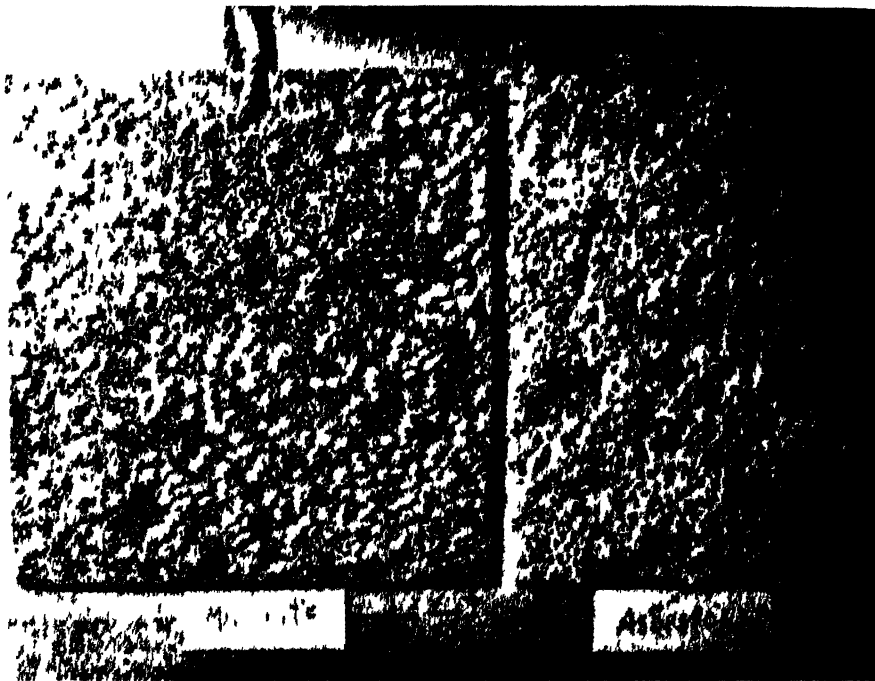


FIGURE 2. Accumulation of fouling organisms on masonite and asbestos after 90 days' exposure at Belle Isle, Miami Beach, Florida

Intact galvanizing on iron was very resistant to marine life. No barnacles were obtained on zinc, on the experimental antifouling paint 7C or on rosin vehicle, a common paint component.

Materials with hard non-porous and non-fibrous surfaces were in general rather poor collectors of fouling. The best accumulation of sedentary populations was found on surfaces which were porous and/or fibrous. Surface of paints, paint ingredients and linoleum are in general non-porous and non-fibrous. Compared to the size and strength of the barnacle cyprid they are also smooth and hard. The histogram (Fig. 3) summarizes the collective efficiency of the substrates.

Some results were undoubtedly spurious, and these should be noted. Fouling on the antifouling paint 8C which occurred along the edges of the panel was probably

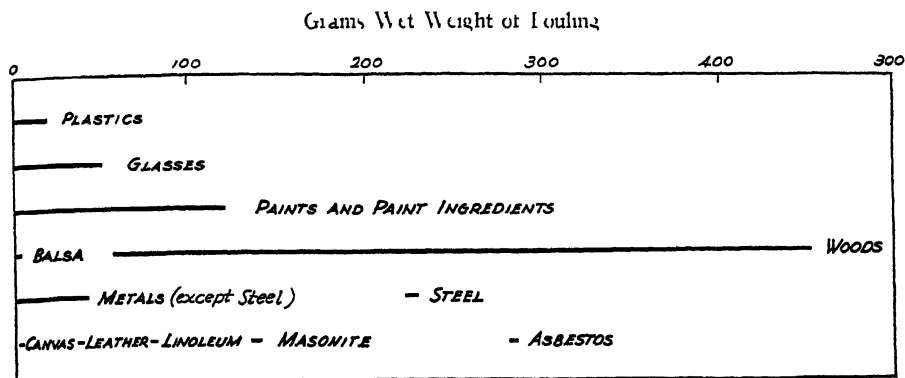


FIGURE 3 Relative amounts of fouling on various classes of materials used as test panels

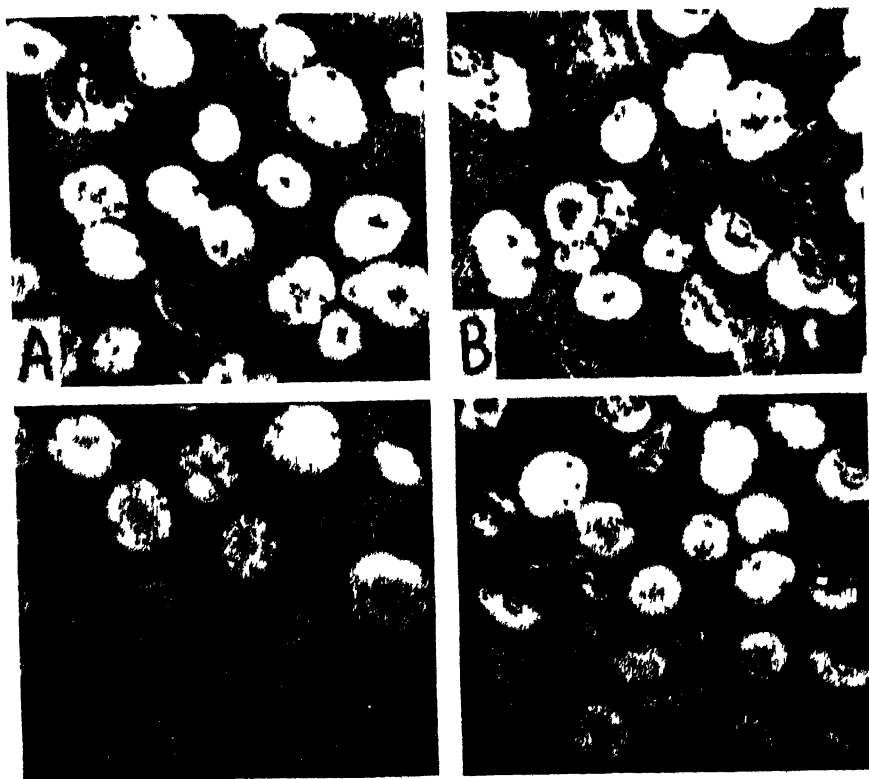


FIGURE 4. Bases of barnacles grown on various substrates A Navy grey B. Antifouling paint 15RC C Ester gum D Anticorrosive paint 42A

due to imperfections of the paint surface. In contrast, 7C which contained less copper was not fouled. Deck canvas and smooth glass both supported a culture of green algae which evidently served as food for fish. Active feeding on these panels unquestionably disturbed other fouling organisms. Balsa wood was apparently sloughing its surface and thus loosening attached forms. In spite of these minor qualifications, the results involve a range of population numbers sufficiently wide to indicate the relative merits of the substrates used.

One of the most interesting of the results was observed when barnacles were removed from the various substrates. Some of the substrates bore barnacles with deeply scalloped margins (Fig 4) instead of the typical smooth edges. These margins suggested that localized irregular marginal growth interruptions had taken place. Such barnacles were collected from:

Spar varnish
Linoleum
Navy grey topside paint (P-50)
Antifouling paint vehicle 15RC
Ester gum paint vehicle
Anticorrosive paint 42-A

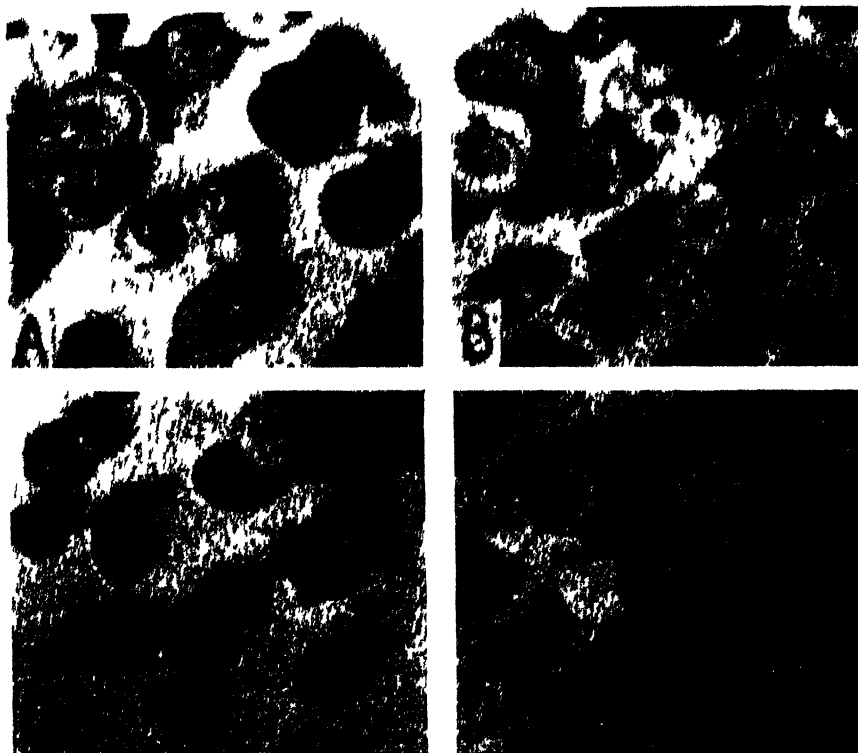


FIGURE 5. Bases of barnacles grown on various substrates. A. Isobutyl methacrylate. B. Plasticel. C. Soft paraffin. D. Ceraloid.

Barnacles growing on soft paraffin had distinctly concave bases. Mosaics of bases with angular margins were typical of barnacles attached to lead but were also found on other overcrowded substrates. It was possible to remove barnacles with intact bases very easily from several materials, including plasticel, ceraloid, and isobutyl methacrylate (Fig. 5). This finding might prove useful in designing experiments in which the minute anatomy of basal structures was to be studied.

SUMMARY

1. Submerged samples of 40 different construction materials were used as substrates for the collection of sedentary populations. The barnacle counts in the populations ranged from 980 on asbestos shingles to zero on zinc and on two paint coatings, after three months' immersion in Biscayne Bay at Miami Beach, Florida.

2. Various surface textures of glass plates were found to exert no significant influence on the accumulation and growth of sedentary marine organisms, although smooth clear glass accumulated smaller populations in the comparatively short exposure periods, 1-3 months.

3. The results suggest that efficiency of a substrate as a fouling collector is in general correlated with porosity of surface or with fibrous nature of surface. Smooth, non-porous, non-fibrous surfaces, especially if also hard, seem to be poor accumulators of sedentary organisms.

4. Further testing of substrates is greatly to be desired in this connection.

REFERENCES

- POMERAT, C. M., AND E. R. REINER, 1942. The influence of surface angle and of light on the attachment of barnacles and other sedentary organisms. *Biol Bull.*, 82 (1): 14.

THE DEVELOPMENTAL HISTORY OF AMAROECIUM CONSTELLATUM II. ORGANOGENESIS OF THE LARVAL ACTION SYSTEM

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INTRODUCTION

The early development of the embryo of *Amaroeicum constellatum* has been presented in a previous paper (Scott, 1945). The accumulation of yolk modifies the pattern of mosaic development characteristic of Tunicates to the extent that gastrulation is accomplished in an atypical manner. Convergence of the cells of the lateral margins of the posterior blastoporal lip is accomplished to the left of the mid-line. The neural plate elongates posteriorly at the place where the lateral blastoporal lips meet and close. The chordal cells are inflected at the anterior lip and lie in the median axis. The potential muscle cells of the morphological right side lie dorsal to the notochord as a result of their growth across the mid-dorsal plane, the muscle cells of the morphological left side lie below the level of the notochord on the curved left side of the embryo. The two groups of muscle cells are separated by the posterior extension of the neural plate.

MATERIALS AND METHODS

Amaroeicum constellatum is abundant along the eastern coast of the United States. The breeding season lasts throughout the summer months. Material for this study was collected at Woods Hole, Massachusetts. The embryos, squeezed from adult colonies, were selected and arranged into a progressive series of stages for study. They were fixed in Bouin's fluid. Some were stained by Conklin's modification of Delafield's haematoxylin, others with borax-carmin, then cleared according to the benzyl-benzoate method described in Romeis' "Taschenbuch der Mikroskopischen Technik." Corresponding stages were sectioned serially, stained in Mayer's or Gallagher's or iron haematoxylin, and counterstained with eosin or triosin. All drawings were made with the aid of a camera lucida. The photomicrographs were made with a Leitz "Macca" camera using Zeiss apochromat, 20 \times , and fluorite oil immersion, 100 \times , objectives with a Zeiss microscope.

Later embryonic development

It seems advisable to present a descriptive series of developmental stages that may be used as points of reference for structures differentiating during the organ-forming period. For convenience the developmental period following gastrulation is divided into four stages; 1) the tail bud stage, 2) early tadpole stage, 3) pre-hatching stage, and 4) the free-swimming tadpole stage. The free-swimming larva

or tadpole has been described thoroughly by Grave (1921) and shall be presented here in brief summary since reference to it is necessary. A short description of the external appearance of these stages will be given first and referred to in subsequent treatment of organogenesis as Stages I, II, III, and IV. The terms, larval action system and adult action system, used by Grave (1935, 1944) will be adopted for the structures functioning during larval life and those functioning during adult life respectively.

The tail bud stage

By the end of gastrulation the embryo is approximately spherical except for a shallow postero-ventral invagination of the ectoderm constricting tail from trunk region. The furrow appearing first on the right side is deeper there, and less deep as it extends to the left side. The tail bud is short and rounded, curving immediately toward the ventral side of the trunk. Through the thin epidermis quadruple rows of large muscle cells can be seen lying dorsal and ventral to the notochord. The neural plate is elevated at the periphery to form the neural groove, enclosing anteriorly a wide depression, the presumptive brain region, posteriorly a narrow, trough-like depression lying to the left of the notochord, the presumptive neural tube (Fig. 1*A*).

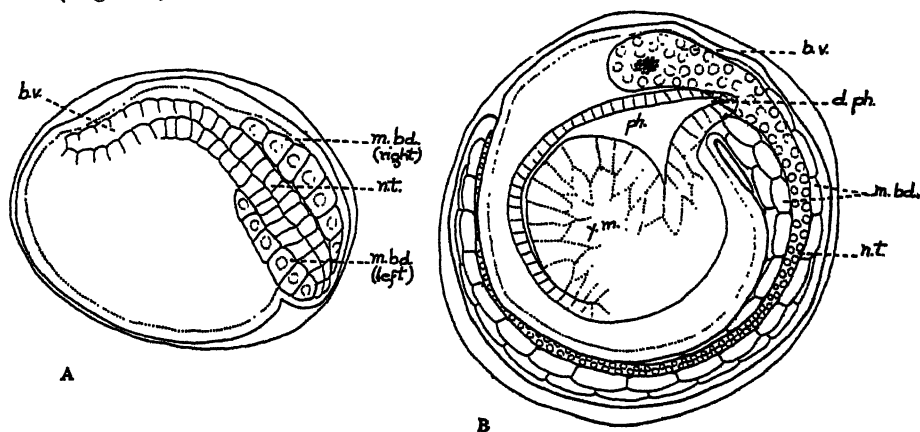


FIGURE 1. *A*. Stage I, embryo before neural folds close. 160 X. *B*. Stage II, early tadpole; beginning of differentiation of digestive and nervous systems. 160 X. *b. v.*, brain vesicle; *d. ph.*, dorsal diverticulum of pharynx; *m. bd.*, muscle band; *n. t.*, neural tube; *ph.*, pharynx; *y. m.*, yolk mass.

A transverse section through the tail bud stage discloses that the embryo is solid. A single layer of definitive endoderm lies under the concave neural plate (Fig. 5*E*). This layer of cells develops from the cells that form the superficial "pseudo-invagination" cavity of gastrulation. The depression closes by a reversal in change of shape of the cells involved rather than by approximation of the lips of the blastopore thus producing a solid archenteron (Scott, 1945). The endodermal cells spread under and anterior to the neural plate. Ventral to them is located the mass of heavily yolk-laden cells derived from the macromeres. Wedged between the

thin ectoderm and the solid endoderm on either side is a mass of mesenchyme, small, polygonal cells with prominent nuclei (Fig. 5E).

Posteriorly the definitive endoderm lies adjacent to the chordal cells which are beginning to interdigitate in the base of the tail bud. The mesenchyme terminates abruptly in this region against the muscle cells of the tail.

Early tadpole stage

The embryo increases in size and acquires the shape that justifies its being called "tadpole." The trunk region elongates slightly in the antero-posterior axis remaining curved at the anterior end. The tail encircles the body meridionally as it grows in length. The embryo is still opaque.

The neural folds are closed, the position of the sensory vesicle being marked by aggregations of black pigment which show through the surface of the body. The neural tube is faintly visible along the side of the tail. More conspicuous are the large muscle cells dorsal and ventral to the prominent notochord which forms the axis of the tail throughout its length. Dorsally, on either side of the sensory vesicle there is a slight ectodermal invagination, rudiments of the atrial chambers. The embryo is confined within a test the cells of which are arranged in a compact layer (Fig. 1B).

Pre-hatching stage

Changes in the external appearance of the later embryo depend on the development of siphons and adhesive papillae and the secretion of a tunic. As body growth continues and organs of the larval action system differentiate, the body becomes transparent except where the mass of yolk is lodged in the pharynx.

The trunk region continues to elongate antero-posteriorly becoming elliptical in shape. Posteriorly the body narrows to the base of the tail; anteriorly it flares in the dorso-ventral axis in relation to the vertical position of the adhesive papillae. Laterally the body is compressed. A thickening layer of tunic invests the entire trunk. It is indented at the junction of trunk and tail and continues over the surface of the tail. The tail encircles the body meridionally being pressed into a groove in the tunic. The tunic of the tail projects laterally into fins.

The sensory vesicle occupies a dorsal position at the posterior end of the trunk. Two masses of pigment project into its cavity. Immediately in front of it lies the elevation of the oral siphon; behind it and on the posterior curve of the body lies the atrial siphon. Much of the internal structure is visible through the tunic and mantle. The incipient adhesive papillae appear as three disc-like projections in verticle series at the rounded anterior end (Fig. 2).

The free-swimming tadpole stage

The trunk of the tadpole of *Amaroecium* at its release measures about 600 micra in length; it measures about 270 micra in depth. The tubular atria with their triple rows of gill slits are pressed into the dorsal pharynx through half of its length posteriorly. An obvious structure in the pharynx is the dorsal, heavily ridged endostyle which seems to rest on the lateral masses of yolk that form the wall of the pharynx. The transparent pericardium occupies a large space below the yolk ante-

riorly in front of the loop of alimentary tract. On the right side of the body the stomach extends along the posterior and ventral curvature of the yolk. On the left the narrow intestine curves along the side of the stomach up to the left atrium where it terminates. The root of the tail lies in the posterior third of the length of the body.

Anteriorly, the adhesive papillae project into the tunic in a vertical row slightly to the right of the median plane. The test vesicles lie loosely within the tunic or many of them, even at the time of hatching, retain a slender connection with the cone or ridge from which they originate. Where the tail is continuous with the trunk the tunic dips down into an abrupt pocket. The epidermis secretes a thin sheath of tunic about the tail. Laterally it expands into wide sail-like fins (Fig. 3).

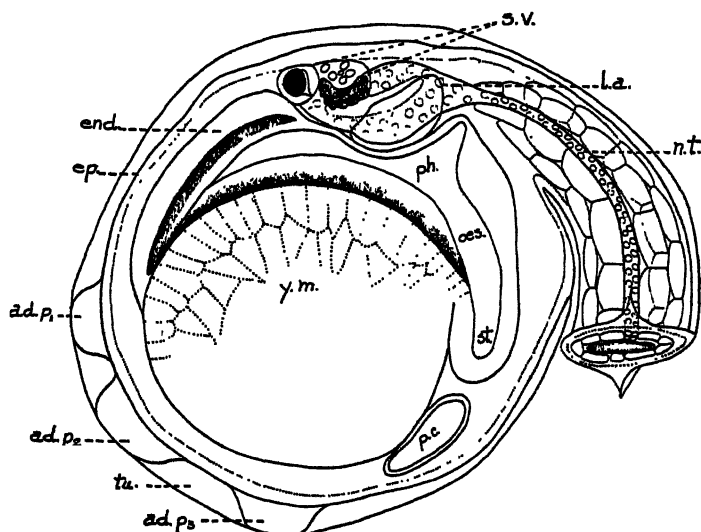


FIGURE 2. Stage III, lateral view of tadpole with incipient adhesive papillae. About 120 X. *ad. p.*, adhesive papillae; *end.*, endostyle; *ep.*, epidermis; *l. a.*, left atrium; *n. t.*, neural tube; *oes.*, oesophagus; *p. c.*, pericardial cavity; *ph.*, pharynx; *st.*, stomach-intestine rudiment; *s. v.*, sensory vesicle; *tu.*, tunic; *y. m.*, yolk mass.

ORGANOGENESIS OF THE LARVAL ACTION SYSTEM

Digestive system

The pharyngeal cavity develops in Stage II by delamination between the layer of definitive endoderm and the mass of yolk-laden cells, appearing first below the brain and spreading from that point (Fig. 1B, 5F, 6E). It extends back to the base of the notochord as an upwardly directed diverticulum. Ventral to the base of this projection a second invagination appears, the rudiment of the stomach and intestine located a little to the right of the median plane on the inner side of the visceral ganglion (Fig. 2, 6F).

The pharynx deepens in Stage III encroaching upon the mass of yolk cells. Gradually thin septa of epithelium divide the yolk mass into four compact longitudinal columns, the two on each side being continuous at the bottom. The central

two are lower than the outer two, thus providing greater depth for the limited pharyngeal cavity (Fig. 6A, F). This supply of nutritive material in the pharynx remains to be digested during the active life of the larva and throughout the critical period of metamorphosis. All other tissues lose their meager supply of yolk almost entirely, leaving their cytoplasm clear.

Along the roof of the pharynx, anterior to the place of origin of the oral siphon, the epithelium rises up into a double fold enclosing the endostyle, restricted to the dorsal side above and between the lateral masses of yolk and passing to the anterior end of the yolk mass (Fig. 2, 6A). Before the tadpole is released from its test, the cells in the floor of the groove develop long cilia. The pharynx grows out above and below the atrial sacs, bringing the mesial atrial and lateral pharyngeal walls into intimate contact (Fig. 6B).

Due to the combined activity of atrial and pharyngeal epithelia, three horizontal rows of gill slits are formed, each consisting of seven or eight perforations. The

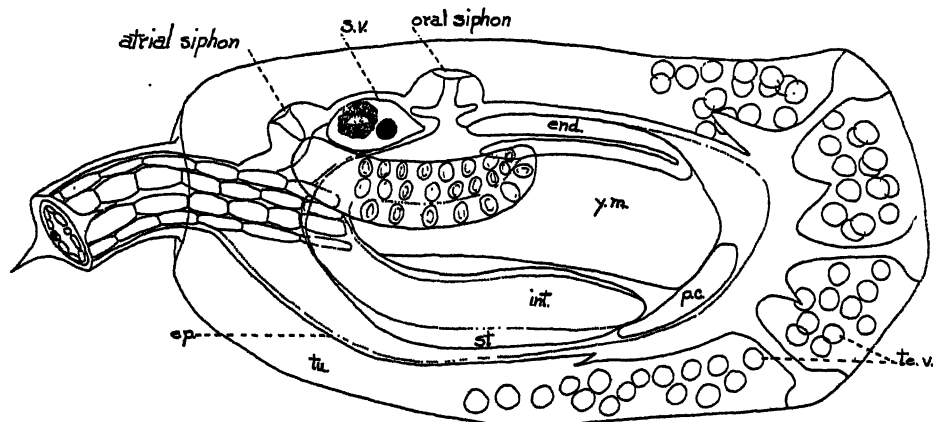


FIGURE 3. Stage IV, tadpole at hatching. About 120 \times . *end.*, endostyle; *ep.*, epidermis; *int.*, intestine; *p. c.*, pericardial cavity; *st.*, stomach; *s. v.*, sensory vesicle; *te. v.*, test vesicles; *tu.*, tunic; *y. m.*, yolk mass.

bordering cells of each gill develop a heavy brush of cilia, precocious equipment from the functional point of view. Even though the mouth breaks through to the branchial chamber, the tunic fills up the oral and atrial siphons until metamorphosis is completed.

The rudiment of the oesophagus grows forward along the curvature of the yolk and dilates to form the stomach. The diverticulum extends to the midventral region of yolk where it turns sharply upon itself and continues backward as the slender intestine. With a gradual slope upward the intestine retraces the course of the stomach on its left side terminating ventral to the posterior end of the left atrium (Fig. 3, 6B). Later the anus opens into the atrium here.

There are no cilia evident in the intestine or stomach during this period of development. The wall of the stomach is thicker than the wall of the remaining parts of the digestive tract although the alimentary epithelium, throughout its length, consists of a single layer of cells.

With rapid general growth of the body, the loop of intestine and stomach increases in length anteriorly, extending through the posterior half of the body cavity below and behind the yolk-laden pharynx (Fig. 3). The pericardium lies directly in front of it. Between the arms of the loop posteriorly are lodged the bases of the axial organs of the tail.

Atrium—During Stage II the atrium or peribranchial sac appears as a pair of ectodermal invaginations, one on either side of the sensory vesicle (Fig. 6E). At the place of its origin the neck of each depression constricts and separates from the surface.

In the transition from Stage II to Stage III, the atria, in contact with the lateral endodermal wall of the pharynx, grow in an anterior direction only, with the result that the atrial chambers are horizontal capsular cavities located dorsally, one on either side of the pharynx (Fig. 6B). They extend through the posterior two-thirds of the trunk, curving gently upward posteriorly where they grow towards each other and unite behind the pharynx (Fig. 3). The atrial siphon opens through the dorsal wall of this connecting canal between the two cavities.

The atrial walls are characteristically thin and the cells lose their intercell membranes. Occasional yolk granules are scattered through the cytoplasm. During Stage III the gill slits perforate the walls in three horizontal rows on the inner side in direct contact with the wall of the pharynx. The lowermost row develops first, the atrial and pharyngeal fusing first in these regions. The slits number between seven and nine in each row. Later in the free-swimming period the cells bordering the gill aperture produce long cilia. The endoderm has no part in atrial formation except insofar as the gill slits are the product of joint activity of atrial and pharyngeal walls (Caullery, 1895).

Oral and Atrial Siphons—Late in Stage III the dorsal ectoderm in front of the sensory vesicle thickens and invaginates, pushing the endoderm of the pharynx before it. The circle of epidermis around the invaginated area becomes elevated, giving the oral siphon a crater-like appearance (Fig. 6B). The floor of the invagination thins out in a flat layer against the pharyngeal roof with which it is in contact. The lower part of the cavity projects outward from the center and produces a ring-shaped extension on the mouth opening. The oral cavity assumes the shape of a flask with a long neck and a flattened base (Fig. 3). Into this ectodermal cavity, or stomodaeum, the hypophysial duct opens, just before hatching of the tadpole. Although the oral plate breaks through late in the tadpole's development, the tunic fills up the stomodaeal portion and prevents the passage of both food and water during larval life.

The atrial siphon, like the oral, is formed by ectodermal invagination. The thickened mantle is elevated, raising the siphon above the level of the rest of the mantle in knob-like fashion (Fig. 6C). The floor of the invagination fuses with the dorsal wall of the connecting arm of the atrium. The atrial siphon is situated on the downward curve of the dorsal surface just posterior to the sensory vesicle and anterior to the insertion of the tail (Fig. 3, 6G, H). The epithelial lining of the oral and atrial siphons projects into each opening at several points forming small tentacles. The mesenchymatous muscles in the mantle in this region provide the contractile elements that control the apertures when the siphons begin to function.

Heart and pericardium

Towards the end of Stage III, the endodermal cells extend completely around the yolk mass as a definite epithelium. Mid-ventrally it evaginates into the body space and constricts off from the yolk epithelium. The bladder-like vesicle is the pericardium which invaginates mid-dorsally into an inner enclosed vesicle, the heart. The cells lose their inter-cell membranes and the nuclei bulge irregularly in both cardinal and pericardial walls (Fig. 6A). The heart does not develop beyond this point at present, the circulatory system not functioning during larval life.

The nervous system

The neural folds of Stage I close in the early phase of Stage II thus forming the hollow nervous system typical of chordates except in one point, the curving of the neural tube through 90° to the left of the brain region. The anterior portion of the nervous system produces the sensory vesicle with its sensory organs, the hypophysis, definitive ganglion, and the so-called subneural gland. The intermediate part including the origin of curvature and a small contribution from the brain region gives rise to the visceral ganglion and the spinal enlargement, the posterior part becomes the neural tube.

The cavity of the brain region is slightly dilated and its wall uniformly thick. The neural tube consists, in section, of four cuboidal cells surrounding a small lumen (Fig. 4C). Cell membranes in both regions are distinct at this stage, the nuclei are large and contain heavily staining nucleoli. The cytoplasm is reticular in appearance and has occasional yolk granules.

During Stage III the brain vesicle differentiates into two structures, the sensory vesicle in the entire right side and the rudiment of the hypophysis on the left posterior side (Fig. 4A, 5A). The vesicle expands, its walls becoming thin; the rudiment of the hypophysis remains small with thick walls. This secondary cavity is separated completely from the sensory vesicle at the region of evagination but their walls remain attached throughout subsequent development (Fig. 5B, C).

The sensory vesicle—Two sensory structures develop in the sensory vesicle, the statolith and the eye. The left posterior wall of the vesicle thickens, the right wall expands dorsally and laterally; all the cells lose their inter-cell membranes. The left wall of the cavity remains thick and constitutes the sensory ganglion of the brain. One cell on the ventro-anterior wall projects into the cavity and large pigment granules are deposited in its cytoplasm; these coalesce to form the statolith (Fig. 4B, 5B). In Stage IV the statolith is a spherical mass of pigment confined within the cell membrane and attached to the ganglionic wall by a stout stalk, the remaining part of the cell (Fig. 4D, 5C, D).

A group of cells situated dorso-laterally at the left posterior limit of the vesicle initiates the development of the eye by the deposition of pigment granules of much smaller size than those that form the statolith. Absence of cell membranes makes it difficult to ascertain the number of cells that participate in this activity. The pigment is deposited in the shape of a cup, its concavity facing dorso-laterally and to the right within the vesicle. Three ganglionic cells which retain their membranes fill up the concavity in series. They secrete globules of liquid which increase in size both by the gradual addition of the secretion and by the fusion of globules. The globules of liquid form the so-called lens cell (Fig. 4A, B, D, 5D). The nuclei

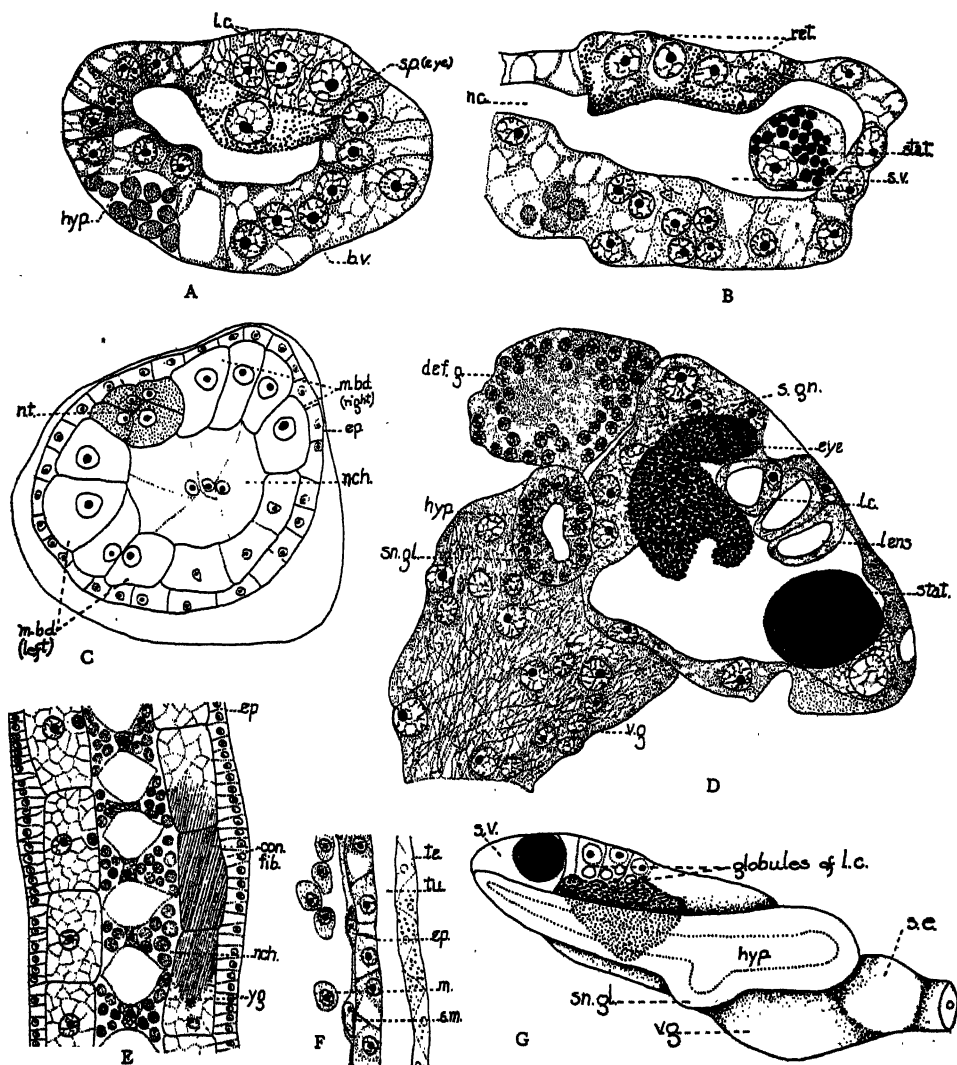


FIGURE 4. A. Transverse section through brain after the neural folds close, Stage II. 750 \times . B. Longitudinal section through brain of same stage. 750 \times . C. Cross section through tail of tadpole, Stage III. 300 \times . D. Section through brain of tadpole just before hatching, oblique to include both sensory organs. 750 \times . E. Longitudinal section through tail of tadpole in Stage III. 300 \times . F. Section through epidermis and test of Stage III. 750 \times . G. Reconstruction of brain and related structures of Stage III, viewed from left side. 300 \times . *b. v.*, brain vesicle; *con. fib.*, contractile fibrils; *def. g.*, definitive ganglion; *ep.*, epidermis; *hyp.*, hypophysis; *l. c.*, lens cell; *m. bd.*, muscle band; *n. c.*, neural canal; *nch.*, notochord; *n. t.*, neural tube; *ret.*, retinal cells of eye; *s. gn.*, sensory ganglion; *sn. gl.*, subneural gland; *stat.*, statolith; *s. c.*, sensory cell; *s. e.*, spinal enlargement; *s. m.*, smooth muscle cells of mantle; *s. p.*, sensory pigment; *t. c.*, test cells; *tu.*, tunic; *v. g.*, visceral ganglion; *y. g.*, yolk granules.

which at first occupy a central position in the cells are pushed to the periphery as the lenses, increasing in size, come eventually to monopolize the entire cell.

The pigment granules of the eye always remain discrete, not coalescing as do those of the otolith. Extending through the concentrated pigment are small rods of clear cytoplasm. They run from the base of the cup back towards the ganglion. Seven or eight of them may be seen in embryos of Stage IV that are mounted in a mixture of benzyl-benzoate and oil of wintergreen.

The hypophysis—The rudiment of the hypophysis early in Stage III appears as an extension or small evagination of the brain cavity (Fig. 4A, 5A). The cells retain their membranes, their nuclei are smaller than those of the adjoining part of the brain. Histologically they present the appearance of epithelial tissue. Upon its separation from the primary cavity during Stage III it elongates antero-posteriorly along the left side of the sensory ganglion (Fig. 4G). In Stage IV it ends blindly at the posterior wall of the oral siphon. Later these walls fuse and the hypophysis communicates with the posterior region of the stomodaeum, extending along the side of the ganglion with a gentle slope upward as far as the atrial siphon where it terminates blindly. The floor of the duct, corresponding in position to the region of the eye, deepens abruptly (Fig. 4D, G). The ventral wall of the pocket becomes slightly thicker, the indentation with its thickened floor constituting the subneural gland. Hjort (1896) reviews the opposing views concerning this structure in the early works on Tunicates.

The definitive ganglion—By a proliferation of cells in the mid-region of its roof in Stage II the hypophysial duct produces an oval mass containing small nuclei similar to those in the hypophysial duct itself. The cell membranes disappear and the nuclei wander out toward the periphery where they collect in several rows with the granular cytoplasm concentrated in the center (Fig. 4D, 4G, 5C). This part of the nervous system, the definitive ganglion, persists through metamorphosis and together with the hypophysis gives rise to the permanent nervous system of the adult.

Visceral ganglion—The visceral ganglion originates in that part of the neural plate that curves toward the left in Stage I. The lumen is obliterated, the large nuclei migrate to the periphery leaving the medulla mass of interlacing fibrils and granules (Fig. 4D). The visceral ganglion lies posterior to and ventral to the sensory vesicle. Dorsally where it merges with the sensory ganglion, it exceeds the sensory vesicle in diameter but it gradually diminishes in diameter towards the base of the tail where it is continuous with the neural tube. At the junction there is a slight enlargement called the spinal enlargement (Fig. 4G). The neural tube retains its lumen. It runs through the length of the tail to the left of the notochord. In Stage IV a single nerve emerges from the visceral ganglion on its right side just below the hypophysis. It runs anteriorly and sends out branches to the smooth musculature of the mantle.

THE NOTOCHORD

At the end of the gastrulation period the chordal cells lie under the posterior part of the neural plate. Anteriorly adjacent to them are endodermal cells; dorsally, the potential muscle cells of the right lateral margin of the blastopore; ventro-laterally, the potential muscle cells of the left lateral margin of the blastopore. Posteriorly the chordal cells extend into the rudiment of the tail.



FIGURE 5. *A*. Transverse section corresponding to Figure 4*A*. 225 \times . *B*. Transverse section through brain of Stage III, hypophysis separated from brain vesicle. 650 \times . *C*, *D*. Sections through sensory vesicle and definitive ganglion of Stage IV; oblique, thus including both sensory organs. 650 \times . *E*. Transverse section through Stage I; anterior region. 150 \times . *F*. Transverse section through early Stage II, neural folds closed. 150 \times . *G*. Longitudinal section through adhesive papilla of Stage III. 650 \times . *d. e.*, definitive endoderm; *def g*, definitive ganglion; *gl. c.*, gland cells; *hyp*, hypophysis; *mch*, mesenchyme cells; *n. p.*, neural plate; *stat.*, statolith.

Some of the endodermal cells of the yolk mass lie along the right side of the chordal cells and when the tail is constricted from the trunk region these cells form the loose column of caudal endoderm. In Stage IV little of it remains (Fig. 4*C*, 6*B*).

In Stage I the notochordal cells begin to shift in position. They interdigitate into a row of disc-shaped cells occupying the central axis of the short tail. The cells

resemble the endodermal cells of the yolk mass in possessing delicate membranes, nuclei smaller than those of adjoining muscle cells, and yolk granules.

During Stages II and III the notochord elongates as the tail lengthens. The chordal cells lengthen; the inter-cell membranes separate from each other converting them into hour-glass shaped cells with the nucleus resting in the constricted neck between the peripheral masses of protoplasm (Fig. 4E).

In Stage IV the cell halves separate completely giving the chord the appearance of a tube with a scalloped lining. The proximal end retains its relationship with the hinder end of the pear-shaped mass of yolk between the atrial cavities and the arms of the digestive tract (Fig. 6C, H). Distally it corresponds in length to the neural tube and tail muscles.

Muscle cells of the tail

Mesoderm differentiates into three structures of the larva, one of which is restricted to the larval action system, two of which function in both the larval and adult action systems. The former includes the muscles of the tail, the latter the muscles of the mantle and mesenchymatous connective tissue in the body cavity. The asymmetry of the posterior lip of the blastopore at the end of gastrulation (Stage I) places the muscle cells of the right side dorsal to the chordal cells and to the right of the neural plate at its posterior end, the muscle cells of the left side to the left of the posterior neural plate but ventral to the notochord (Fig. 4C). Each band is made up of four cells in fairly regular rows.

In Stage II the myoblasts are the most prominent cells in the body because of their size and heavy membranes. Each cell contains a large faintly reticular nucleus with a conspicuous nucleolus. The deeper cytoplasm is grossly reticular and retains an occasional yolk granule (Fig. 6D).

In Stage III the peripheral cytoplasm elaborates in its cortex, in a slightly spiral direction, along the longitudinal axis rows of contractile fibrillae composed of minute granules so distributed that they resemble the individual myofibrillae of striated muscle of the higher chordates (Fig. 4E, 6D). The myofibrillae are continuous from one cell to another throughout the length of the muscle bands. Grave (1921) has described this in the free-swimming tadpole of *Amaroecium*. The bases of the muscle bands, like that of the notochord in Stage IV, are located well within the posterior part of the trunk just behind the mass of yolk (Fig. 6H).

Muscles of the mantle

In the late embryonic period (Stage III) many of the mesenchyme cells located directly under the ectoderm unite end to end to form the smooth fibres of the mantle (Fig. 4F). One set of such muscle fibres radiates from each of the siphons. The other set encircles the trunk obliquely from the dorsal to the ventral side.

Mesenchyme of the body cavity

In Stage I two compact lateral masses of mesenchyme cells lie pressed tightly between the nutritive endoderm and shallow ectodermal cells. The one on the right side is disposed more dorsally than the one on the left side (Fig. 5E). They extend from the posterior muscle cells towards the anterior end of the body.

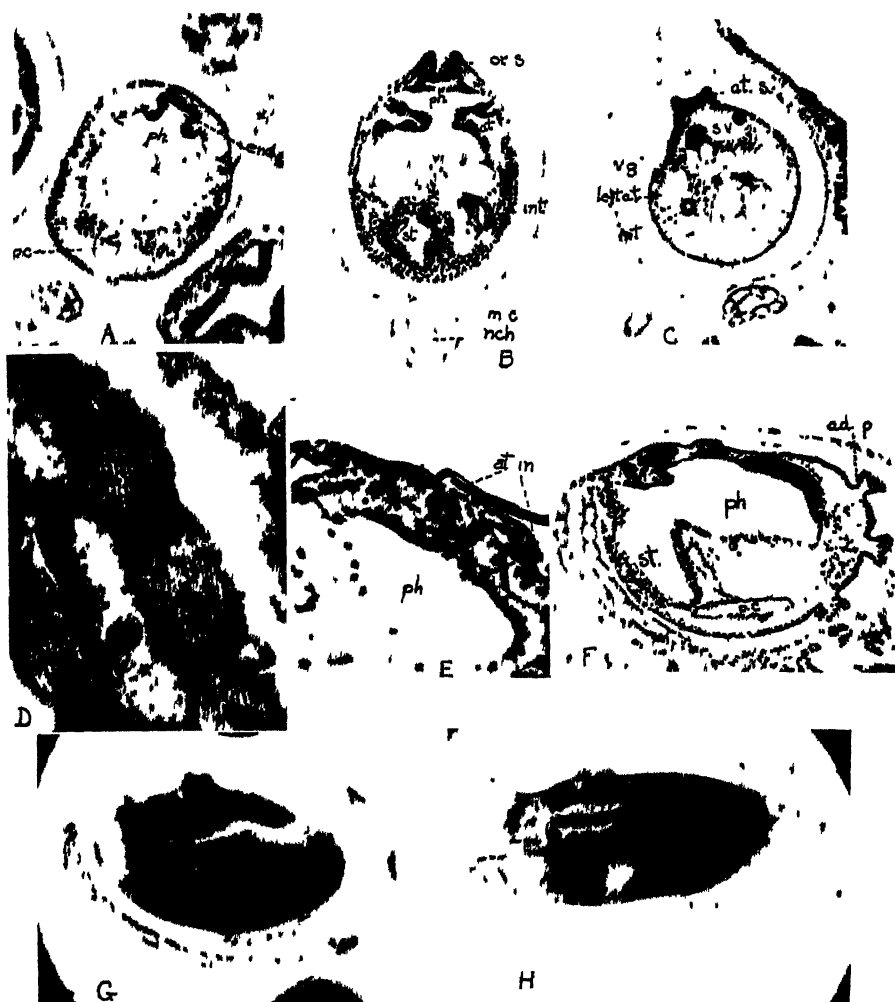


FIGURE 6. *A, B, C.* Transverse sections through tadpoles of Stage IV; *A*, anterior. *B*, In region of oral siphon. *C*, In region of atrial siphon. About 200 \times . *D*, Section through part of muscle band; middle cell through center of muscle cell, lateral cell through peripheral cytoplasm where myofibrillae are formed. About 850 \times . *E*, Transverse section through Stage II to show atrial invaginations. 300 \times . *F*, Longitudinal section through Stage III. About 150 \times . *G*, Tadpole just before hatching, chorion not ruptured. About 250 \times . *H*, Tadpole at hatching. Note insertion of notochord at posterior end of trunk. About 250 \times . *ad. p.*, adhesive papilla; *at. s.*, atrium; *at. m.*, atrial invagination; *at. s.*, atrial siphon; *end.*, endostyle; *int.*, intestine; *m. c.*, muscle cell; *nch.*, notochord; *or. s.*, oral siphon; *ph.*, pharynx; *p. c.*, pericardial cavity; *st.*, stomach; *s. v.*, sensory vesicle; *v. g.*, visceral ganglion.

In Stage II both masses of cells multiply and spread out under the ectoderm in all directions except posteriorly. A small amount of mesenchyme is found in the tail, probably derived from the cells in the mid-region of the posterior lip of the blastopore. Being crowded together the cells appear angular in section. The nuclei are relatively large (Fig. 4F, 5G).

During the transition from Stage II to Stage III growth of the body and absorption of the yolk effect a separation between the epithelial cells of the epidermis and the endodermal cells (Fig. 5F, 6F). As the body cavity enlarges the cells round up, separate from each other, and wander freely about, dividing frequently and eventually filling up all available space except in the area around the base of the tail (Fig. 6A, B, C).

Other mesenchyme cells assume stellate shape and send out long slender strands of protoplasm by means of which they form a reticulum of mesenchymatous tissue. This is the nearest approach to a coelomic epithelium that is found in Tunicates with the possible exception of the perivisceral cavity of *Ciona*.

The mantle and tunic

The epidermis in Stage I is a layer of thin cells small in surface view dorsally where they adjoin the neural plate, larger towards the ventral body region. In Stage II the cells are of uniformly small size and cuboidal in section except where they invaginate to form the atrium and are columnar in shape. In surface view all present the characteristic polygonal arrangement of epithelial tissue.

During Stage III the protoplasm becomes vacuolated medially, the nuclei being pushed to the periphery where the cytoplasm is more granular (Fig. 5B, 6E). The epidermal cells grow thinner as development progresses and the inter-cell membranes disappear. When the epidermis has assumed the characteristic appearance of the Tunicate mantle in Stage III it secretes a thick layer of structureless tunic. Occasional cells of the test of the ovum are trapped in the clear tunicin, the greater number, however, being pushed with the test ahead of the tunic (Fig. 6C). The tunic is grooved where it is secreted about the tail and when the tail is released, with the disappearance of the test, the groove remains in evidence marking the embryonic position of the tail.

Derivatives of the epidermis

Adhesive papillae—A conspicuous feature of the *Amaroecium* larva is a vertical row of three adhesive papillae at the anterior end (Fig. 3, 6F, G, H). Each papilla first appears early in Stage III as a local thickening of ectoderm forming a pad of columnar cells. The cells at the periphery of the thickened pad form a stem which increases in length as the tunic thickens, the whole organ becoming goblet-shaped. It retains its connection with the body cavity through its slender hollow stem (Fig. 6G, H). The papillae extend through the thickness of the tunic and are exposed at its surface. Cells that constitute the functional portion become vacuolated and reticular proximally and toward the center of the cup, where the long cells converge, they produce secretion granules which lodge in the concavity of the papilla (Fig. 5G). The bordering epidermis surrounds the disc forming a thin layer over the cup-shaped depression. During the free-swimming life of the larva

the secretion granules are converted into a viscid substance by means of which the tadpole becomes attached. The entire glandular structure is of ectodermal origin. Grave (1921) from his study of the fully formed tadpole supposed that mesenchyme cells gave rise to the glandular portion of the papilla. Mesenchyme cells wander from the body cavity into the hollow stalk but they are not incorporated into its structure. The tail encircling the body crowds the papillae a little to the right of the sagittal plane thus adding to the asymmetry of the larva. The three papillae cannot be homologized with the tactile papillae of *Botryllus*, which are integral parts of the peripheral nervous system. Here they serve only as gross organs of attachment.

Test vesicles—During Stage III, when the adhesive papillae are differentiating, the test vesicles originate as numerous small ectodermal evaginations in four distinct regions at the anterior end of the trunk. Two groups, separated from each other by the median papilla, are directed forward. The dorsal group is derived from a short ridge extending in the direction of the oral siphon. The ventral group, below the ventral papilla, is derived from a long ridge extending posteriorly through about a third of the length of the trunk (Fig. 5, 6G, H). The vesicles themselves originate as independent hollow slender projections of the ectoderm. The attached end of each evagination becomes narrow, finally constricting off and severing its connection at the base. Frequently this separation is not effected by the time of hatching of the vesicle still being attached to the epidermis by their stalk-like bases. When detached the slightly pear-shaped vesicle rounds up and becomes a sphere consisting of a single layer of cells which lose their definition on the proximal side where they are extremely thin.

The use of the word "test" in connection with these vesicles is unfortunate. The chorion of the egg of *Tunicates* is called the test and the cells that either lie freely in the enclosed liquid or are resolved into pavement epithelium are called the test cells. The tunic of the tadpole is a purely ectodermal derivative. The tunic of the adult colonies being the product of secretory activity of these vesicles, the vesicles should, with greater accuracy, be called the "tunic vesicles."

SUMMARY

1. The digestive system of *Amaroecium* lacks an open archenteron at the end of gastrulation. The pharynx appears as a narrow incision with a thin roof and heavy floor. An oesophageal evagination differentiates into stomach and intestine.
2. Heart and pericardium originate from the floor of the pharynx.
3. Atrium and siphons are ectodermal structures that become associated with the digestive system.
4. The nervous system consists of a sensory vesicle enclosing two sensory masses of pigment, a hypophysis lying beside two sensory ganglion, a visceral ganglion descending laterally to the neural tube which lies to the left of the notochord throughout the length of the tail.
5. The notocord is derived from chordal cells invaginated at gastrulation. Its cells become vacuolated. The notochord is confined to the tail and posteriormost region of the trunk.
6. Muscle cells differentiate from mesodermal cells of the blastoporal margins. Asymmetry of the blastopore places the cells of its right margin dorsal to the noto-

chord, the cells of the left margin ventral to the notochord. Each band of muscle cells consists of four longitudinal rows. Cells separate from the two lateral masses of mesenchyme and move into the body space of the developing tadpole. They give rise to muscles of the mantle.

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COMPARATIVE SENSITIVITY OF SPERM AND EGGS TO ULTRAVIOLET RADIATIONS

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The sperm of the sea urchin are more sensitive to ultraviolet radiations than the eggs when the effectiveness of the rays is compared by the retardation of cleavage of unexposed eggs fertilized with irradiated sperm on the one hand and of irradiated eggs fertilized with unexposed sperm on the other (Giese, 1939c). It would be interesting to know whether sperm are generally more susceptible to these radiations than eggs; therefore, the experiments were repeated on a number of marine forms. It is also desirable to find an explanation for this differential susceptibility in those cases where it occurs. Insight might be gained by determining action spectra for the sperm and egg, therefore the relative efficiency of action of different wave-lengths of ultraviolet light in retarding cleavage of irradiated eggs and of eggs fertilized with irradiated sperm was determined as described below.

MATERIALS AND METHODS

Arbacia punctulata, *Nereis limbata*, *Chaetopterus pergamentaceus*, and *Mactra* sp. were studied at Woods Hole, Mass. *Strongylocentrotus franciscanus* and *S. purpuratus*, collected at Moss Beach, and *Urechis caupo* collected at Bolinas Bay, California, were used at Stanford University, and *Dendraster excentricus* and *Parteria miniata* were studied at the Hopkins Marine Station, each type of egg being used during the active breeding season.

The methods for studying the eggs were similar to those previously described (Giese, 1938). Except for the work on the action spectrum, the mercury argon discharge tube which emits about 85 per cent of its light at λ 2537 Å was used and the light intensity was measured with a Hanovia Ultraviolet Meter (No. 949). The dishes were kept in running sea water to attain a lower temperature than that of the room. The work on the action spectrum was done with a mercury arc and a natural quartz monochromator and the intensity of the light was measured with a thermopile as in previous studies (Giese, 1938). The eggs were kept in dishes in moist chambers and in a constant temperature room at 15° C.

Sperm were used in dilutions of between 1 : 200 and 1 : 1,000 of the spawn. Such dilution is necessary because in denser suspensions ultraviolet light is completely removed by the sperm first reached. Irradiated sperm lose their fertilizing power rapidly, therefore they must be used soon after exposure (see Hinrichs, 1927, for studies on inactivation).

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EXPERIMENTAL

Comparison of various eggs

A summary of the general results obtained with all the eggs studied will be found in Table I. Not all the eggs respond to ultraviolet radiations in the same way. Thus cleavage of eggs of *Arbacia* and *Strongylocentrotus* is merely slowed up but remains normal after small and medium dosages so that comparisons of the effects of various dosages and wave-lengths is relatively easy. Abnormalities only appear after larger dosages. In *Urechis*, *Nereis*, and some of the other eggs the threshold for abnormal development is relatively low. While per cent of abnormal development could be used for analysis of effects of radiations, it would be much more difficult.

It is readily apparent that with regard to ultraviolet susceptibility, there are two types of sperm: in the Echinoderms, especially *Arbacia* and *Strongylocentrotus*, the

TABLE I

Comparative action of ultraviolet radiation¹ on eggs and sperm of various marine animals

Species	Effects on eggs	Effects on sperm
<i>Strongylocentrotus purpuratus</i>	Delay just noticeable after about 100 ergs/mm. ² ; will develop even after 4,000; after 8,000 ergs/mm. ² become quite abnormal.	Noticeable delay ² even after 10-20 ergs/mm. ² Marked retardation as dosage above this is used.
<i>Arbacia punctulata</i>	Noticeable delay after 200 ergs/mm. ² but even after 2,000 ergs/mm. ² plutei, normal but smaller than controls, develop from eggs. After 4,000-8,000 ergs/mm. ² eggs are quite abnormal.	Noticeable delay even after less than 50 ergs/mm. ² After 250 ergs still develop larvae but after 500 quite abnormal. Even after 4,000 ergs/mm. ² sperm activate eggs.
<i>Dendraster excentricus</i>	Slight delay after 1,600 ergs/mm. ² ; strong after 6,400; quite abnormal after 25,000 ergs/mm. ²	Slight delay after 200 ergs/mm. ² ; abnormal after 800 ergs/mm. ² .
<i>Urechis caupo</i>	Some delay after 200 ergs/mm. ² Marked injury with abnormal cleavage after 5,000 ergs/mm. ²	Marked abnormalities after 200 ergs/mm. ²
<i>Chaetopterus pergameneus</i>	Slight delay only after about 4,000 ergs/mm. ² ; after 16,000 ergs/mm. ² still cleave but much delay and many cytolize.	Slight delay after 2,000 ergs/mm. ² ; killed after about 8,000-16,000 ergs/mm. ² .
<i>Nereis limbata</i>	Even after 4,000 ergs/mm. ² develop with little delay to the trochophore stage; after 8,000 ergs show delayed cleavage.	Slight delay between 4,000-8,000 ergs/mm. ² 8,000 kills most sperm.
<i>Mactra</i> sp.	Very slight delay after 500 ergs/mm. ² ; striking after 4,000-8,000.	Delay after 250 ergs/mm. ² and progressive delay thereafter.

¹ The measurements with the Hanovia meter are accurate to about 10 per cent as checked by thermopile measurements in one instance.

² Amounting to 15-30 minutes delay at the third cleavage of eggs fertilized with such irradiated sperm. Marked delay means a delay of an hour or more.

sperm are much more sensitive than the eggs; in the worms such as *Urechis*, *Nereis*, and *Chaetopterus* the sperm is slightly if at all more sensitive than the egg, as judged by cleavage delay.

Such a lack of differences in susceptibility of the gametes might be more apparent than real. It is possible that when there is little or no cleavage delay following fertilization of an egg with an irradiated sperm, the sperm may be serving only to activate the egg to haploid parthenogenesis. Eggs of *Arbacia* and of *Chaetopterus* were, therefore, fertilized with sperm treated either to a small dosage or to a medium dosage of radiations and at appropriate intervals samples were fixed in Bouin's fluid and stained with iron hematoxylin. Although the preparations were

ACTION SPECTRUM FOR RETARDATION OF CLEAVAGE

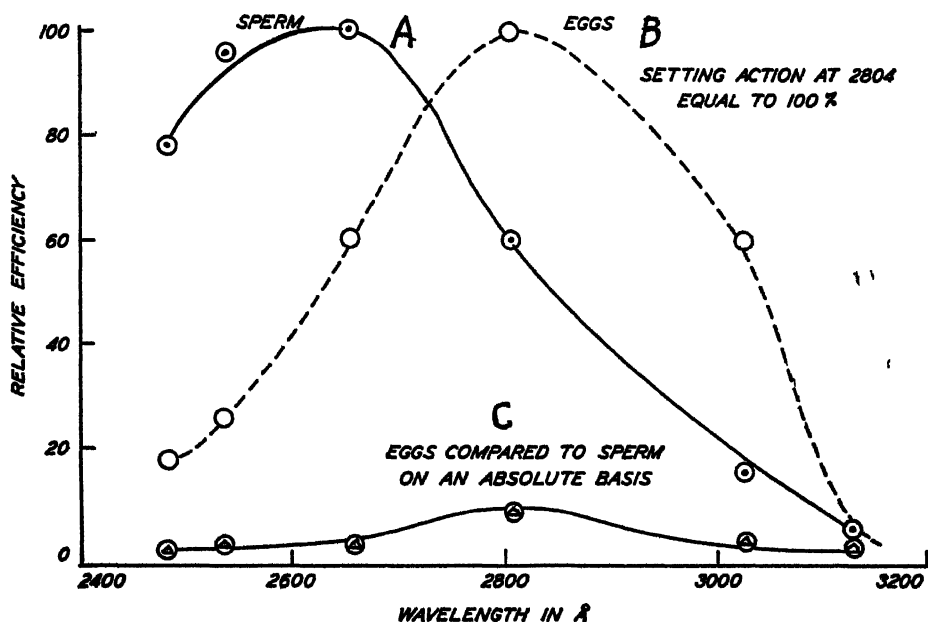


FIGURE 1. Action spectra for retardation of cleavage of eggs fertilized with irradiated sperm at A and for irradiated eggs at C. At B the data for the eggs are compared on a relative basis setting the value at λ 2,804 Å as 100 per cent efficient. See text below.

not entirely satisfactory, evidence for pronuclear fusion was observed in both cases. No lagging or disintegrating sperm were observed in the cytoplasm of either egg. Since neither cytological nor physiological evidence suggests parthenogenesis, it seems likely that for the dosage ranges tested the delayed cleavage follows fusion of the gametic nuclei. The difference between the two types of sperm must lie in some other factor. Possible explanations will be considered in the discussion.

The data in Table I show that the threshold for effects on cleavage is quite different for eggs of different species. Thus *Strongylocentrotus*, *Arbacia*, *Mactra*, and *Urechis* eggs are retarded after brief exposures to ultraviolet as compared to

Nereis, Chaetopterus, and Dendroaster. Whether this is due to mere physical screening by some inert materials in the egg or to differences in concentration of some light sensitive materials is not known.

Action spectra for egg and sperm

If irradiation of the nucleus alone causes retardation of division of the cell, the same action spectrum should be found for egg and sperm; that is, there should be no qualitative difference in effectiveness of different wave-lengths even though the general susceptibility of the sperm is greater. If elements in the cell other than the

ACTION SPECTRA FOR SPERM AND EGG AND PROTEIN ABSORPTION

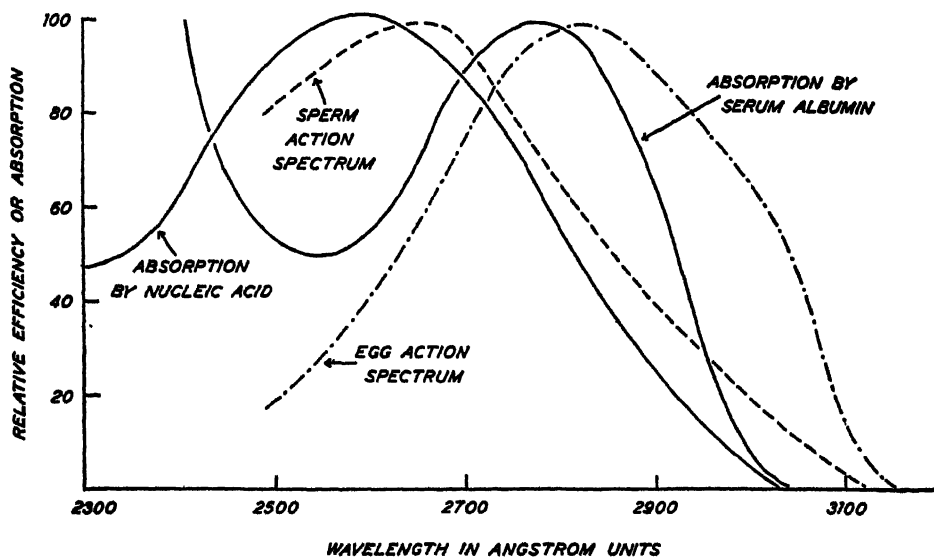


FIGURE 2. Comparison of the action spectra of Figure 1 with absorption spectra of nucleic acid and serum albumin. Data for nucleic acid from Caspersson (1938), for proteins from Smith (1929). Note that the action spectrum for the egg is quite different from the absorption spectrum for albumin at both ends.

nucleus are involved the egg may show an action spectrum different from that of the sperm.

The methods employed for the studies at different wave-lengths are similar to those already described elsewhere (Giese, 1938, 1939c), therefore, only the briefest mention need be made of them. The irradiated eggs are fertilized with normal sperm. The rate of division is then determined by observing for percentage of cleavage every 15 minutes. The times at which the eggs reach the 2, 4, 8, 16, and 32 cell stages are recorded and the number of cleavages is plotted against the time after fertilization and compared with the control. The increase in time required to reach the third cleavage is taken as a measure of the retarding action of the radi-

ations. The retardation is then plotted against dosage. From such curves for each of the wave-lengths the dosage required to bring about a given retardation can be determined. For Figures 1 and 2 the reciprocals of the relative amounts of energy at different wave-lengths required to produce a retardation of division by 1.5 hours were determined. In Figure 1 at *A* and *C* the sperm and egg are compared on this basis and a great difference in susceptibility between the gametes is evident. In *B* the data for the eggs are compared amongst themselves on a relative basis setting the action at λ 2,804 Å as 100 per cent efficient.

The shape of the curves indicates that different materials are being affected in the two cases, since the action spectrum is considered to be a measure of the absorption by the active constituent. To see if the absorbing materials can be identified the absorption spectra for serum albumin and nucleic acid are given in Figure 2. It is apparent that the action spectrum for sperm matches the absorption spectrum for nucleic acid better than the absorption spectrum for albumin; the reverse is true for the action spectrum of the egg. Since the simple proteins and nucleoproteins are the major structural constituents of the cell and none of the other organic or inorganic constituents have very specific absorption, the resemblances while imperfect are indicative of absorption by these two classes of compounds in the action of ultraviolet radiations on the gametes.

DISCUSSION

The occurrence of a differential susceptibility of gametes with the sperm more sensitive to ultraviolet light than the egg as first found in the sea urchin, *Strongylocentrotus purpuratus*, was verified on *Arbacia* and *Dendraster* and in preliminary trials on *Pateria* and *S. franciscanus* but not on *Urechis*, *Chaetopterus*, and *Nereis*. In the latter forms the sperm appears to be only slightly more sensitive than the egg (Table I). The former group of species belongs embryologically to the radially-cleaving, indeterminate egg type, the latter group to the spiral determinate type. In addition, the radial eggs used here are mature or nearly so at the time of shedding whereas the spiral eggs are generally immature. An illustration of the difference in response to ultraviolet light, depending on this difference in organization is seen in the local "burns" occurring in the spiral eggs. Thus a *Nereis* egg given a unilateral dosage of between 8,000–16,000 ergs/mm.² may develop apparently normally except on the burned surface which appears blistered. A *Strongylocentrotus* egg on the other hand unless given a large dosage of light will show general effects distributed throughout the retarded egg. However, it is not possible to say which features of the organization account for the difference in sensitivity of the eggs and sperm of the two groups.

One might envisage that in eggs the retarding effects of radiation on cleavage are due to the inactivation, by substances formed during irradiation, of some catalyst which is necessary for the reactions involved in cleavage. In one group of eggs perhaps the catalyst is present in excess of that necessary for a characteristic rate of cleavage, the rate being controlled by some other limiting factor, in the other it is present in just adequate concentration and itself constitutes the limiting factor. Even a considerable dosage of radiations will not reduce the concentration of catalyst below the critical level in the first case but will readily do so in the second. In the first case no cleavage delay would be expected until very large dosages of radi-

ations had been administered, in the second the cleavage should be affected after very small dosages. One would have to assume that irradiated sperm on penetrating unirradiated eggs introduce similar cleavage-inhibiting substances acting on the catalyst as those formed in the irradiated egg. In this case also the effect on cleavage should depend upon the amount of catalyst present in the egg—if in excess, the cleavage should not be easily inhibited, if limiting, the reverse should be true. We should expect both sperm and egg to be relatively insensitive to the radiations in the former and this is found in most spirally cleaving eggs.

Against the above postulation is the fact that the action spectrum for sperm resembles nucleoprotein absorption while for the egg it resembles simple protein absorption indicating two different ultraviolet absorbing materials in the gametes by which the cleavage-retarding effect is produced. It is possible that absorption by both of these types of proteins leads to the formation of toxic photoproducts which inhibit the same catalyst. It is also possible that the toxic substance is much more rapidly formed by the nucleoproteins, but the necessary assumptions strain the imagination.

It should be pointed out that the retardation of the early cleavage is only the initial effect of the radiation. If the delayed effect could be studied we might find that the recovery from injury to the egg would resemble absorption by nucleoprotein, indicating a more lasting injury to the nucleus than to the cytoplasm, as is the case for division of *Paramecium* (Giese, 1945a). Because the number of cells cannot be satisfactorily determined in the later cleavages such experiments were not attempted with eggs.

The action spectrum obtained for the egg is similar to that observed for "cytoplasmic" effects such as increased time of ciliary reversal, retardation of excystment, immobilization of cilia, and prevention of hatching of eggs. The action spectrum for the sperm resembles that for "nuclear" effects such as recovery of paramecia from sublethal effects, bactericidal and fungicidal effects and the production of mutations (see Giese, 1945b, for references). It is interesting to note the difference between the action spectrum for retardation of cleavage of the egg and for activation studied by Hollaender (1938). In the latter case no action was found until about λ 2,650 Å and the effectiveness of the light increased as the wave-length decreased. The mechanism of action of the light must be different in these two instances. The action spectrum data thus lay the foundation for further analysis of the effect of these radiations upon gametes.

SUMMARY

1. The action spectrum for the retardation of division of eggs fertilized with irradiated sperm resembles the absorption of ultraviolet light by nucleoproteins.
2. The action spectrum for retardation of division of irradiated eggs of the sea urchin resembles absorption by simple proteins like albumin except that at the short wave-length end there is no increase in action at λ 2,483 Å where absorption shows a definite upswing.
3. The absolute amount of energy required to affect division to the same extent by affecting the sperm is very much less than that required to affect eggs.
4. Other Echinoderms tested show a similar difference in susceptibility of eggs and sperm: *S. franciscanus*, *Arbacia punctulata*, *Dendraster excentricus*, and *Parthenocladia miniata*.

5. Animals other than Echinoderms tested did not show as striking a difference between susceptibility of eggs and sperm: *Urechis caupo*, *Macra* sp., *Chactopterus pergamentaceus*, and *Nereis limbata*.

6. In the eggs listed in paragraph 5, determinations are made more difficult by the tendency for the eggs to show irregular cleavage rather than retarded cleavage as the dosage increases. Such irregular cleavage occurs in Echinoderm eggs as well but the threshold is higher.

7. If both eggs and sperm of the sea urchin are irradiated the effect on the rate of division is less than the sum of the effects which would be expected on each of the gametes alone. However, the percentage of abnormal cleavage greatly increases.

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OBSERVATIONS ON THE FUNCTIONING OF THE ALIMENTARY SYSTEM OF THE SNAIL *LYMNAEA STAGNALIS* *APPRESSA* SAY

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INTRODUCTION

Although records exist of functional studies on the alimentary system of Basommatophora as far back as the early eighteen hundreds, the detailed story of the course and ultimate fate of food in the alimentary tract and the simultaneous movements of the tract is thinly scattered and far from complete. In the more recent emphasis placed on some gastropods because of their importance as vectors of parasites of man, domestic animals, wild game, and fish, it is vitally important that the normal physiology of the system most frequented by these parasites be better known.

It is the purpose of this paper to integrate the previous work on the physiology of the alimentary system of *Lymnaea stagnalis* and allied forms (suborder Basommatophora, order Pulmonata) with original research on the same system in *L. s. appressa* Say. The basic morphological (Carriker, 1945) and histological (Carriker and Bilstad, 1946) studies on this system in *L. s. appressa* have been completed and are in press. All terms used in this research have been described in these two papers.

L. s. appressa has been selected for this research because it is a representative vector and because of its excellent response to laboratory culture, its relatively large size (maximum shell length, 62.5 mm.) as compared with other fresh water pulmonates, its short life cycle, and its relatively thin semitransparent shell and semitransparent tissues. Snails used in the research were cultured entirely in the laboratory. They were grown through many generations in large battery jars and fed on lettuce and cooked "cream of wheat" cereal. The water in the jars was aerated by means of a small Marco air pump (Noland and Carriker, 1946). The original snails were collected in Fox Lake, Wisconsin, in 1939. Parasite-free cultures (especially of trematodes) from the original snails were obtained by the isolation of the egg mass soon after oviposition in separate aquaria. Each new culture was started in this way rendering transmission of infection very improbable. Detailed examination of succeeding generations has not disclosed parasites.

This work was carried out at the University of Wisconsin (1939-1943) under the stimulating guidance of Prof. L. E. Noland, whose advice, encouragement, and friendly cooperation were much appreciated.

HISTORICAL REVIEW

Scanty observations on the function of the anterior part of the alimentary tract of *Lymnaea* were given by Semper (1857), Geddes (1879) and Moquin-Tandon (1885); more detailed information was given by Amaudrut (1898), Pieron (1908)

and by Baecker (1932). The stomach region was investigated by Gartenauer (1875), Moquin-Tandon (1885), Colton (1908) and Heidermanns (1924). These experimental contributions of Colton and of Heidermanns, particularly of the latter, are noteworthy. The liver has been the object of most of the physiological work although the research has usually been incidental to that on the stylomatophoran *Helix*: Barfurth (1880, 1881, 1883a and b), Frenzel (1886), Cuénot (1892), Enriques (1901, 1902), Faust (1920), Peczenik (1925) and Krijgsman (1928). Only the investigation of Peczenik is exclusively on *L. stagnalis*.

EXPERIMENTAL METHODS AND RESULTS

Lymnaea physiological salt solution

The study of the living system has required the development of a physiological salt solution which will approximate the ionic and osmotic balances of the blood of *Lymnaea* more closely than do such commonly used solutions as Ringer's. On the basis of incomplete data given by Duval (1928) on *Lymnaea* and by Bernard and Bonnet (1930) on *Helix* on the molecular concentration of blood, the following solution was developed for *L. s. appressa*:

NaCl.....	2.0	gms.	per	liter
NaHCO ₃	2.0	"	"	"
KH ₂ PO ₄	0.1	"	"	"
MgCl ₂	0.3	"	"	"
CaCl ₂	0.3	"	"	"

This solution consists of 0.47 per cent salts and gives a pH of approximately 7.8. After about a week considerable precipitation of CaCO₃ occurs, although this seems to have no noticeable effect on the isolated organs. The vas deferens was used in testing the solution and was found superior to the heart for this purpose. The vas deferens, terminal preputium and prostate gland were removed under the physiological salt solution from the cephalic hemocoel without bruising. This portion of the reproductive tract is in part a strong muscular tube which is easily excised and maintains a continuous squirming motion as long as the tissues are alive. It continued squirming for about 66 hours in the solution described above. A Ringer's solution of 0.7 per cent salts keeps it moving for about 12 hours, although at a much reduced rate.

Hydrogen ion concentration

The first work on the estimation of the pH of the alimentary tract of a fresh water snail seems to be that done by A. H. Rosenbloom on *L. s. appressa* in his bachelor's thesis in 1942 (unpublished) in this laboratory. He has kindly consented to the incorporation of his results in this paper. His method was essentially the colorimetric one employed by Yonge (1925): fluids from the various lumina of the alimentary tract of the snails under variable feeding conditions were pressed out onto paraffined plates and thoroughly mixed with indicators (brom-thymol blue, neutral red, and methyl red). The colors were compared with those of indicators freshly prepared in buffered solutions checked on a Coleman pH electrometer. The results are given in Table I:

TABLE I

pH of the contents of various lumina of the alimentary tract of L. s. appressa

Organ	Average pH	Maximum and minimum pH	Number of tests	Number of snails
Buccal cavity		Same as water in external medium.		
Procsophagus	6.9	7.2-6.3	10	10
Postcsophagus	7.2	7.6-6.6	10	10
Gizzard and crop	6.4	7.2-6.3	12	12
Pylorus	6.6	7.0-5.8	10	10
Intestine	7.1	7.8-6.2	35	15

Enzymes

Preliminary tests were made for non-purified cathepsin, pepsin, trypsin, and amylase. The tests for the proteinases were made according to the methods of Anson (1938), Bradley (1938) and Folin and Ciocalteu (1927); those on amylase, by the iodine test of Hawk and Bergeim (1937). Semi-micro technics were applied to large numbers of the excised organs.

Maximum catheptic activity (at pH 3) over a ten-day period was found in the liver. That occurring in the buccal mass and gizzard and other portions of the alimentary system was not significant as compared to that in the liver. In an effort to determine to what extent cathepsin might be secreted from the liver, gut fluid from which the amebocytes had been centrifuged was tested. Under the conditions of the experiment, at least, no cathepsin was found in the gut juice. In some tests tryptic activity was found in the salivary glands. A very active amylase, optimum pH 7, was present in the salivary glands and in the liver.

The only investigation of the hydrolytic enzymes of the alimentary system of the Basommatophora reported in the literature is that by Heidermanns (1924). He described a positive test for cellulase present in the digestive juice of the stomach organs (crop, gizzard, and pylorus) of *L. stagnalis*.

Ciliation

Ciliary currents were studied by the injection of fine carmine suspensions in *Lymnaea* physiological salt solution through various portions of the exposed tract, by application of carmine particles to the epithelium of the opened tract and by placing small bits of gut wall in a carmine suspension on an uncovered microscopic slide under high magnification. In some dissections the undisturbed food particles were seen passing through various portions of the excised gut on the natural ciliary currents.

No work has been performed previously on the ciliation of the alimentary system of the Basommatophora. Merton (1923) in his research on the external ciliation of pulmonates included a brief study of the ciliation of the hepatic ducts of *Helix*.

The entire alimentary system of *L. s. appressa*, with the exception of the gizzard and portions of the buccal cavity, is ciliated (see later in this paper), Figures 3, 9, and 11.

Muscular activity

The activity of the alimentary system was observed under binoculars through the transparent walls of normal living young snails and in adult unanesthetized snails opened under *Lymnaea* physiological salt solution. The independent activity of the radula over the odontophore was clearly observed and conclusively verified by watching snails under the binocular under the following conditions: snails deprived of food for a day were placed in a finger bowl of well aerated water to which had been added strips of lettuce (1–2 mm. wide). A Petri dish was floated over the lettuce and the water. As the snails crawled upside down under the glass, feeding on the lettuce, the action of the radula and mouth parts was clearly visible under a strong beam of light.

Sand in the gizzard

In order to check the experiments of Heidermanns (1924) and to add additional information on the role of sand in the comminution of food by the gizzard of *L. s. appressa*, the following experiments were devised.

Sixteen adult snails were placed in each of four aerated aquaria containing a one-half inch mesh wire platform over the bottom. By means of this contrivance the feces were removed from the vicinity of the snails soon after defecation. To three of the aquaria the following foods were added respectively: (1) cooked "cream of wheat," (2) filter paper, and (3) lettuce. (4) No food was added to the fourth tank. (5) A fifth tank was assembled as a control without the wire platform and with lettuce and sand. One snail from each aquarium was killed daily and opened immediately. After ten days the following was disclosed: eight of the forty-three experimental pulmonates contained no sand in the tract, thereby showing that it is possible to rid completely the tracts of a few of the snails of sand; however, there was extensive variation in the ability of the different snails to retain sand. As the quantity of sand in the gut decreased, the snails consumed less food, until in the absence of sand in the tract, no food was ingested and the guts became void of food material and feces. The different diets indicated no significant difference in their respective values as sand eliminators. Sand was found most abundantly in the gizzard lumen, then in decreasing amounts in the crop and retrocurrent passage of the pylorus (anatomical terminology has been described elsewhere, Carriker, 1945). After the quantity of sand in the lumen of the gizzard reached a certain low level, it was retained with surprising tenacity for many days. The material in the fecal pellets of the control snails, particularly of the gizzard residues, was markedly brown and more thoroughly triturated than those of snails with sand-free diets.

In a second set of experiments snails approximately 10 mm. in length were placed in a one-quarter inch mesh wire basket suspended in a large laboratory snail stock tank. The feces, propelled by the sluggish circulation of the water in the tank, passed out of the basket. All lettuce placed in the basket was carefully washed to remove sand. The experiment was continued for several months. In spite of precautions, small quantities of fine sand were always present in the tracts of some of the animals; however, this did not seem to be enough for proper trituration as many of the snails died abnormally at an early age and none reached the normal adult size of the control snails in the tank outside the experimental basket. There

is unquestionably a vital need for the presence of at least a limited quantity of sand in the gizzard of these snails for sufficient breaking down of the food.

These results are in agreement with the findings of Heidermanns (1924) and of Colton (1908). Heidermanns accidentally discovered that the only way to entirely remove the sand from a live snail was to cause it to hibernate, in which state it emitted the total contents of the tract. Colton noted that in the presence of sand the plant food was cut to pieces by *L. columella*, but that in the absence of sand it went unmolested.

Digestive cell ingestion

By the use of a method patterned after that of Peczenik (1925) the ingestion of particulate food by the digestive cells was investigated. White of egg was strained through cheese cloth. Carbon (lamp black) was ground into the egg albumen and the mixture was thoroughly beaten. This was steamed to a stiff mass and fed to snails starved for a few days. After feeding commenced, the snails were opened every other day. Indigestible residues within vacuoles in the digestive cells as well as similar residues in the fecal pellets showed the presence of very minute particles of carbon, particles not present in the control snails. The indigestible residues in the digestive cells appeared very similar to the albumen passing down the intestine in the gizzard residues.

Fecal rhythms

Some information was gathered on the rhythms of the liver and of the gizzard by a study of the rate and extent of passage of the various fecal strings. The fecal pellets of a 40 mm. snail were observed daily for twenty-four days. The animal was isolated in a two-liter glass jar over the bottom of which was placed a parafined one-half inch mesh galvanized metal screen, so that all fecal pellets fell to the bottom of the jar and could not be reconsumed. The mollusc was fed lettuce on which was sufficient sand for the needs of the stomach region. Three egg masses were oviposited by the snail, and it added 2 mm. of shell during the twenty-four day period. Upon dissection at the end of the experiment the animal appeared normal in all respects. For the first ten days the pellets were collected and examined microscopically every few hours during the day; during the latter part of the experiment they were collected every twelve hours. Numerous examinations were made of fecal pellets from the stock snail tanks to corroborate the findings on the experimental snail.

PHYSIOLOGY OF THE ALIMENTARY TRACT

Buccal mass and esophagus

L. s. appressa is primarily an herbivore. In the laboratory it may complete its life cycle on lettuce alone and in its natural state feeds on the aquatic vegetation of its surroundings. Specialization of the alimentary system (Carriker, 1945) has been in keeping with a plant diet. However, animal food is also consumed as has been observed by Walter (1906) and by seven other authors cited by him. Repeatedly in this laboratory *L. s. appressa* has been observed to eat the bodies out of the

shells of dead snails in the aquaria. Biochemical tests disclose the presence of some tryptic activity in the secretion of the salivary glands.

Pieron (1908) has found in *L. auricularia* and *L. stagnalis* that there is a total absence of food discrimination in the buccal mass and that their feeding is a reflex which keeps the radula working most of the time. The only portion of the body showing any discrimination is the anterior surface of the foot which contains faintly sensitive chemoreceptors. In aquaria in this laboratory *L. s. appressa* rasps much of the time, whether on lettuce or over the newly cleaned glass surface of its tank. However it does also pass through regular "resting" periods in which no rasping occurs. In the rasping stroke the radula passes first to one side and then to the other describing a broad feeding track.

Feeding can be followed clearly in normal immature "albino" *L. s. appressa* (a strain with very little dark pigment) feeding on a "cream of wheat" food mixture blackened with lamp black. This can be seen to pass as far as the stomach region. On the protractor stroke the radula cups to an elongated spoon-shaped trowel about one-half the width of the upper mandible, and working against this, cuts out long narrow bits of food. Each denticle is sharp so the concerted action of the numerous denticles on the radula, sliding independently over the odontophore, provides an effective cutting-rasping apparatus. The food bits are pushed back through the dorsal food channel to the rear of the buccal cavity which dilates to receive them. The tip of the radula closely appresses to the dorsal wall of the buccal cavity in its rearward passage, as attested by the jagged pattern of the dorsal chitinous surface. The buccal aperture constricts strongly and rapidly after the receding radula. Some bits of food are dropped and remain in the dorsal food channel for the next rearward swing of the food-laden radula. Several food bits clump in the rear of the buccal cavity prior to being forced down the esophagus. The radula functions principally in cutting pieces of food of suitably small dimensions for convenient transport through the anterior portion of the alimentary tract; it does not triturate the food to any considerable degree.

Only the posterior third of the buccal cavity is ciliated. These cilia and those in the densely ciliated esophagus beat strongly posteriorly, bearing food bits from the rear of the buccal cavity to the crop.

In connection with the functioning of the buccal mass, refer to a previous paper (Carriker, 1945) for the names, origin, and insertion and relations of the muscles and parts of the mass. The muscular activity of the buccal mass is divisible into four major synchronous movements: (1) opening and closing of the oral aperture and consequent spreading and approximation of the mandibles and lips, as well as dilation and contraction of the circular muscles about the anterior portion of the buccal cavity, (2) backward-forward and simultaneous elevator-depressor movements of the odontophore, with some slight turning of the odontophore on its longitudinal axis and some movement to the right and to the left, (3) movement of the radula and radular sac over the cartilage, and (4) backward-forward and simultaneous elevator-depressor movements of the entire buccal mass. Consequently there exist in the buccal mass three intrinsic focal points about which the majority of the muscles radiate: (1) the oral aperture, (2) the odontophoral cartilage, and (3) the radula and the radular sac.

The activity of the odontophore with respect to the remainder of the buccal mass may be arbitrarily divided into four phases, and described as follows: (1) the *quies-*

cent stage in which the odontophore lies at rest in the rear of the buccal cavity with its longitudinal axis in a dorsoventral position. (2) The *protracting stroke* in which the proximal end of the odontophore swings in an arc of about 130° from its basal position to a point where it lies above the plane of the distal end, which then is in a position to pass partly out of the buccal cavity, bringing the radula against the substratum. At the beginning of this stroke the odontophore assumes a horizontal position as a result of the lowering of the distal end by contraction of the dorsal odontophoral flexor muscle, and a simultaneous raising of the proximal end by strong contraction of the posterior jugalis muscle. The oral aperture and the anterior portions of the buccal mass dilate to permit partial protrusion of the odontophore through the mouth; the labial retractors, suboral dilators and dorsomandibular dilators spread the mouth. The extrinsic postventral levators and posterior jugalis further raise the rear of the buccal mass so that the distal tip of the odontophore is directed towards the oral aperture, to which it seems to be guided principally by the action of the dorsal odontophoral flexor muscles. The inframedian radular tensors draw the radula over the distal end of the cartilage to the point where most of the radula outside the radular sac lies on the under side of the horizontally inclined cartilage, and the collostylar hood lies just behind the distal crest of the cartilage. The combined action of the radular sac and cartilage tensors holds the radula tautly drawn over the cartilage in readiness for the rasping stroke. Contraction of the intracartilage tensors adds considerably to the rigidity of the cushion under the radula. As Woodward (1895) points out for *Natalina caffra*, the fibers of the cartilage act in much the same way as the intrinsic muscles of the human tongue and in contraction cause an elongation and consequent slight protrusion of the radula. The pressure of the blood in the odontophoral sinus probably provides further turgidity. Contraction of the extrinsic as well as of the intrinsic protractor muscles brings the odontophore to the substratum. (3) In the *rasping stroke* the distal tip of the odontophore is drawn over the substrate in a licking motion. The radula, independent of the principal motion of the cartilage under it, is itself simultaneously slid quickly backward most of its length over the cartilage by the action of the heavy supralateral and supramedian radular tensor muscles. The odontophore is aided by contraction of the extrinsic preventral levator muscles which pull the anteroventral floor of the buccal cavity forward and upward. As the mouth opens during the previous stroke, the cutting distal margin of the dorsal mandible is turned partly forward by contraction of the dorsomandibular dilators and possibly the posterior jugals. Thus as the radula rasps forward it makes connection with and scrapes past the inner side of the dorsal mandible, much as two jaws would come together, so that the snail when feeding on thin portions of lettuce actually "bites" off pieces with each rasping stroke. It is only when feeding on thicker foods that true "rasping" comes into play. The dorsal mandible is governed by the dorsomandibular approximator muscle. The lateral mandibles afford mechanical protection to the sides of the mouth, and close in medially after the radula and under and behind the dorsal mandible. (4) The *retractor stroke* returns the odontophore to the resting condition, and completes the cycle, by action of the extrinsic retractor muscles and the supralateral and supramedian radular tensors and relaxation of the protractors. The oral aperture is closed after the receding odontophore by action of the labial sphincter and the mandibular approximator muscles; the buccal cavity, by a contraction of the buccal sphincter and related mus-

cles of the walls. In assuming the resting position, the radular sac is depressed behind the cartilage and the radula rests principally behind the vertically arranged cartilage so that the ventral tip of the sac projects slightly below the level of the buccal mass. As observed by Amaudrut (1898) for *Lymnaea*, the ventral wall of the buccal cavity between the esophageal ledge and the collostylar hood is also depressed, forming a slight dilation in front of the esophageal opening. As both the oral aperture and the proesophagus are closed during the retractive stroke of the radula, it is likely that this dilation is instrumental in creating a slight vacuum in front of the esophageal opening which aids in disengaging food particles from the radula. The dilation is caused principally by depression of the radular sac and possibly by contraction of the superior suspensor muscle of the radular sac and the hood tensor muscles.

The proesophagus is limited in its muscular activity to slight peristaltic waves proceeding towards the postesophagus; while the latter undergoes pronounced peristaltic activity in either a forward or a backward direction, dilating broadly and contracting its entire length. In dilation it may become so large as to fill much of the cephalic hemocoel of the expanded mollusc. In expansion it is filled with a reddish fluid from the stomach region and food particles.

In the buccal cavity the food receives generous quantities of fluid from the buccal gland cells, a fluid which is probably mostly mucoid in nature, judging from the positive mucicarmine stain and from negative tests for amylase and trypsin. This does not however preclude the possibility of the presence of other enzymes which were not tested for. As food passes under the openings of the salivary ducts it receives mucus, amylase, trypsin, and possibly other enzymes from the salivary glands.

The proesophagus adds more secretion from buccal glands and mucous cells. The postesophagus functions as a temporary reservoir for the retention of food when the crop is full. Being capable of considerable distension, it may retain larger quantities of food than the crop. Digestion begins in the postesophagus because of enzymatic secretions received from the salivary glands.

Stomach region

Comminution of food particles is completed in the crop, gizzard, and anterior portions of the retrocurrent passage of the pylorus. These three organs act as a unit comparable to a grist-mill. The kneading motion of the anterior and posterior gizzard constrictor muscles and the gizzard lobes over the sand in the lumen provides the grinding action. Food bits forced between the sand are soon crushed to minute particles upon which the digestive enzymes may act more efficiently. Two synchronized movements are present in the gizzard. In the first the anterior and posterior gizzard constrictor muscles alternate smoothly in mild contraction, thus mixing and forcing the contents of the gizzard slowly back and forth; in the second, not as frequent as the first, the bulk of the gizzard compressor muscles contract suddenly and strongly, bringing pressure to bear on the contents of the gizzard. The presence of gritty material in the gizzard of the Lymnaeidae has been noted by many: Cuvier (1817), Wetherby (1879), Whitfield (1882), Moquin-Tandon (1885), Colton (1908), F. C. Baker (1900, 1911), and Heidermanns (1924).

In the crop, all ciliary currents lead to the anterior margin of the right gizzard

pad, those on the left side beating ventrad and over to the right (Fig. 3). Thus fine food material accumulates on the right side of the crop at the anterior edge of the right gizzard lobe. The crop receives food from the postesophagus and forces it into the gizzard lumen. When ample sand is accessible to the animal, the crop and anterior portions of the retrocurrent pyloric passage are both filled with it. The walls of these organs act as mechanical obstructions to the open ends of the gizzard lumen and concentrate the pressure of the gizzard musculature upon the contents. They also cooperate in the muscular activity of the gizzard in a unified kneading and a slow rotation of the gritty contents. The retrocurrent passage returns to the crop those particles which have been dislodged from the gizzard contents by muscular movements of the stomach region. In this fashion the contents of the gizzard undergo thorough comminution and partial digestion before the residues are shunted down the procurrent passage to the prointestine.

The epithelium of the stomach region bears a complicated system of ciliary currents (Figs. 1, 2, 3, 9). Cilia in the procurrent passage direct fine particles from the right ventral side of the gizzard cavity to the prointestine. Those in the retrocurrent passage are directed antieriad towards the left side of the gizzard cavity. The dorsal passage bears what in fixed sections appears to be nothing more than a brush border. Even in carmine suspensions under high magnification no distinct current could be detected in it. The cilia on the ventral fold are divided into two distinct functional areas: those on the right half of the fold beat obliquely posteriad and laterally in the direction of the currents in the procurrent passage; those on the left half, obliquely anterolaterad in the direction of the gizzard and the currents in the retrocurrent passage. The currents on the minor fold whip obliquely anterolaterad; those on the medial half of the major fold pass obliquely anterolaterad; while those on the lateral half of the major fold and those on the medial half of the fold adjacent the hepatic vestibule reach posterolaterad. The ciliary currents in the retrocurrent passage are noticeably faster than those in the procurrent passage. Currents on the atrial corrugations run into the incurrent tubule of the cecum. Thus the pylorus in cross section (Fig. 2) is composed of three channels, each with distinct ciliary currents and of three folds which almost meet centrally and whose

EXPLANATION OF PLATE I

(All figures concern *L. s. appressa*)

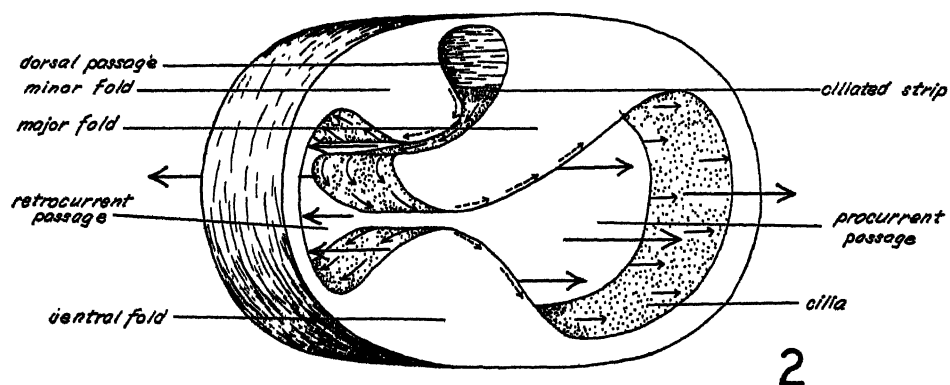
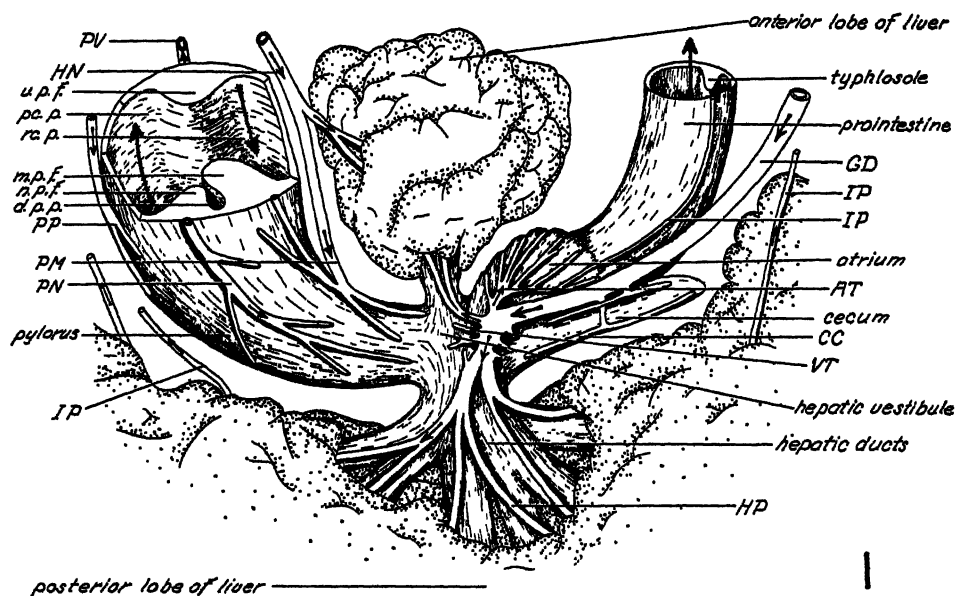
FIGURE 1. Stereogram of the pylorus, hepatic vestibule, atrium, cecum, anterior portion of prointestine, and liver lobes. The vascularization is stressed. (Small arrows indicate the flow of blood in the arteries; large arrows, the direction of movement of the contents of this part of the tract.) $\times 6$.

FIGURE 2. Stereogram of cross-section of the pylorus, taken midway between the gizzard and the hepatic vestibule. The stippled surfaces are heavily ciliated. (The small arrows indicate the direction of the ciliary beat; the large arrows, the direction of passage of material in the pylorus. The arrows with broken stems designate the direction of ciliary beat on surfaces behind the folds.) $\times 25$.

ABBREVIATIONS

AT, atrial artery; CC, cecal artery; *d.p.p.*, dorsal pyloric passage; GD, dorsogastric artery; HN, minor hepatic artery; HP, prohepatic artery; IP, prointestinal artery; *m.p.f.*, major pyloric fold; *m.p.f.*, minor pyloric fold; *p.c.p.*, procurrent pyloric passage; PM, major pyloric artery; PN, minor pyloric artery; PP, propyloric artery; PV, ventropyloric artery; *rc.p.*, retrocurrent pyloric passage; *v.p.f.*, ventropyloric fold; VT, vestibular vascular arborescence.

PLATE I



ciliary currents pass out of the dorsal into both the procurrent and the retrocurrent passages. The major fold in addition bears a thin longitudinal strip of long cilia at its boundary with the dorsal passage. The major and minor folds in the living animal nearly always touch along their crests, so that the fluid contents of the dorsal passage may pass into the two ventral passages but coarse material from the ventral passage may not pass into the dorsal passage. The juxtaposition of the two folds is continued under the hepatic vestibule, where the folds provide a ventral floor to this chamber. At this point the cilia on the folds direct a powerful current out and away from the vestibule, again preventing the entrance of coarse material into the hepatic ducts and liver.

As discovered for *Helix* by Merton (1923), the corrugations of the larger proximal portions of the hepatic ducts of *L. s. appressa* bear two ciliary countercurrents (Fig. 11): the cilia on the crests of the corrugations are long and beat into the liver, those in the grooves are shorter and pass particles in the direction of the hepatic vestibule and into the incurrent tubule of the cecum. The particles in the grooves are quickly entrapped in mucus secreted there and formed into delicate strings. The currents directed into the liver could be traced with certainty only in the large hepatic ducts, although cilia were observed as far as the peripheral follicles in isolated bits of living liver tissue. Yonge (1936) states that in Mollusca where food passes into the liver and waste material out, the ducts are ciliated in such a way that an inward passage exists on one side and an outward one, on the other. Such counter currents could not be determined in *L. s. appressa*.

In the cecum the cilia on the cecal folds beat off the folds into the tubules (Fig. 9); those in the incurrent tubule pass carmine particles directly to the distal end and around this into the excurrent tubule. Here the cilia beat circumferentially, rotating the contents of the tubule along the longitudinal axis. In the continuation of the excurrent tubule across the pyloric wall the ciliary stream is directed towards the prointestine.

The crop, pylorus, liver, and hepatic ducts are as active as the postesophagus. Besides the usual peristaltic movements, they undergo a series of violent alternating pulsations, here designated *pulsatory movements*, in which the crop, pylorus, hepatic ducts, and liver pulsate successively, forcing the fluid contents slowly back and forth in swirling currents. In the pylorus the pulsations commence at a point between the typhlosome and the atrium and pass towards the gizzard. They are of two types: (1) very strong pulsations in which the entire structure contracts and (2) minor pulsations running over restricted portions of the pylorus. In the liver the pulsations pass as far as the terminal follicles. This marked movement is most vividly observed in bits of living liver tissue under high magnification. Individual cells are seen to move against each other by contraction of the thin muscular connective sheet enveloping each follicle. The pylorus undergoes the most pronounced movements and appears to lead the other organs in activity. The incurrent tubule of the cecum is relatively thin-walled and does not appear to undergo peristaltic activity. The excurrent tubule is thicker-walled and has definite peristaltic movement in the direction of the outlet.

It follows then that one of the important functions of the pylorus is that of a filter chamber, separating the digested and the fine, partly digested food particles from the gross material and sand. This is the conclusion which Heidermanns (1924) also reached when he stated that most of the time sand and gross material

are kept from passing into the liver by the pyloric folds. The major and minor folds remain in close approximation along their crests, leaving a narrow slit between the dorsal and the ventral passages which may be called the *pyloric filter*. The cilia on the folds are well developed and beat away from the dorsal passage. During the pulsatory movements of the stomach region only the finest particles and fluid material are permitted ingress to the liver through this filter. The *pulsatory currents*, as these in the gut lumen may be named, are relatively strong and in their streaming between the sand particles and food bits in the gizzard cavity dislodge large particles of food. Those which are carried into the pro- and retrocurrent passages and which are too large to pass through the pyloric filter, become entangled in the ciliary currents of the folds and are carried quickly back to the *left* side of the gizzard lumen by way of the retrocurrent passage. The particles carried into the crop on the forward streaming of the contents are soon entangled in the ciliary currents of the crop and conveyed to the *right* side of the gizzard lumen. Here, then, is a delicate adjustment by which the larger particles dislodged from the gizzard contents are equally redistributed for further grinding within the gizzard.

At certain intervals during the day the pulsatory movements appear to cease and a portion of the residual material and sand in the gizzard pass out through the procurrent passage to the prointestine. The propulsion of *gizzard strings* (Fig. 10), as these residues may be named, through the procurrent passage is very slow and mostly by cilia supplemented by slight peristalsis. Cilia were found active throughout all portions of the alimentary tract whenever opened; no cessation of ciliary activity (as occurs in some lamellibranchs during increase of CO₂ concentration) or reversal of beating was observed. During emission of the gizzard string, the large portion of the ventral pyloric fold which partly occludes the gizzard lumen flattens to enlarge the opening. As suggested by Howells (1942) for *Aplysia*, it appears that the shape and position of the pyloric folds in *L. s. appressa* are partly maintained by blood pressure in the sinuses.

To what extent digestion does occur in the postesophagus, crop, gizzard, and pylorus is questionable. As amylase from the liver and from the salivary glands, trypsin from the salivary glands and cellulase, at least, are present in the gut contents, some food may be partly hydrolized. Part of the remaining available food is reduced mechanically to particles small enough for ingestion by the digestive cells of the liver. The amoebocytes of the gut also appear to aid in digestion. According to Heidermanns (1924) fats and carbohydrates are absorbed in the pylorus by the ciliated cells.

The pyloric filter permits only minute food particles to pass into the liver. Most of the radular teeth which are discarded continuously from the radula throughout the life of the snail (Carriker, 1943a) and grains of sand as large as 90 μ , by reason of the fact that they are considerably heavier than the lighter food particles of the same dimensions, are carried past the cilia by the force of the pulsatory currents. The larger free food particles, especially of lettuce, are very light and are readily barred by the cilia of the filter. In the proximal portions of the hepatic ducts, because of counter ciliary currents, only the finer particles that fall into the grooves of the corrugations can be carried towards the cecum; thus teeth and larger sand grains are held at this point by the ciliary currents of the crests of the corrugations until sufficient fecal material passes out of the liver to carry them with it.

Ciliation of the crests of the corrugations may play a small role in the conduction

of food material into the follicles of the liver, but probably the principal conveyers are the pulsatory currents. Food in solution and in suspension is thus brought to all the internal surfaces of the liver follicles. Larger particles finding entrance through the filter and too large to remain readily in suspension appear to fall to the ductal epithelium. The smaller of these are soon propelled into the grooves of the corrugations. Liberal quantities of mucus are secreted there, trapping the particles in mucous strings which pass towards the cecum, coalescing as they advance into the larger grooves (Fig. 11). From the incurrent cecal tubule the mucoid strings pass around the distal end of the cecum into the excurrent tubule. There the material receives a further transparent layer of mucoid and cementing material and is rotated into a smooth cylindrical continuous string, here designated the *cecal string* (Fig. 10). This, partly by ciliary action and partly by peristalsis, then passes on into the prointestine across the atrium. In snails feeding on green lettuce the strings are a vivid green because of a heavy accumulation of bits of chlorophyll bearing bodies which become entangled in mucous strings in the hepatic ducts. In gastropods fed on a food containing carbon, the cecal strings are a dense black. In animals on a starvation diet, the cecum continues to pass out cecal strings, just as in the feeding animal, but the strings are a mucoid, transparent, milky-white color and much reduced in diameter. It thus would seem that the function of the grooves in the hepatic corrugations and of the cecum is to collect and eliminate those fine particles which pass through the pyloric filter but which are too large to be engulfed by the digestive cells and which are thus mechanically eliminated by a "supplementary filter." Cecal strings pass out continuously, apparently at the same uniform rate and without apparent interruption. They provide a kind of "time clock" by which the rate of passage of the gizzard strings and the residues from the liver can be compared (Fig. 10).

EXPLANATION OF PLATE II

(All figures concern *L. s. appressa*)

FIGURE 3. Ciliation currents of the postesophagus, crop, gizzard, pylorus, hepatic vestibule, atrium, and anterior portion of prointestine. The tract has been slit ventrally and spread. $\times 6$.

FIGURE 4. Irregular blue-green excretion bodies (in vacuoles) taken from the liver string. $\times 500$.

FIGURE 5. Smooth blue-green, or brown, excretion bodies (in vacuoles) taken from the liver string. $\times 500$.

FIGURE 6. "Signet" excretion body (in vacuole) appearing in the liver strings. $\times 500$.

FIGURE 7. Clear nodules found in the liver strings which when pressed out under the cover slip display their crystalline nature. They dissolve in dilute HCl and seem very similar to the calciferous concretions of the vesicular cells of the connective tissue. $\times 500$.

FIGURE 8. Indigestible residues from digestive cell (in vacuole), found abundantly in liver strings. $\times 500$.

FIGURE 9. Ciliation currents of the cecum, which has been opened along the incurrent cecal tubule and spread flat. $\times 6$.

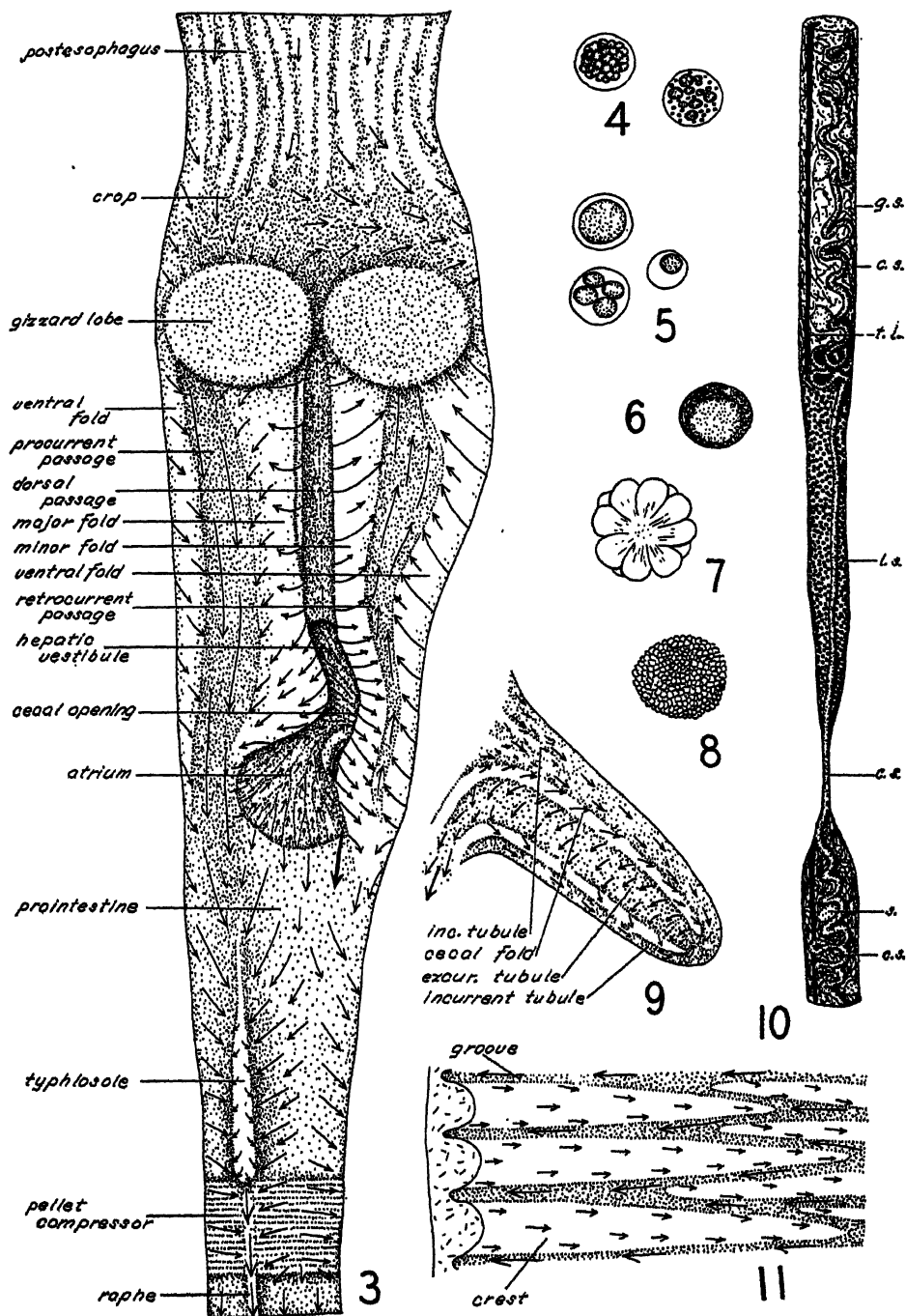
FIGURE 10. Typical fecal pellet, showing the gizzard, liver and cecal strings, and the impression of the typhlosole in the pellet. $\times 6$.

FIGURE 11. Portion of the corrugated epithelium of the hepatic duct, taken at the opening of the duct into the hepatic vestibule. (Large arrows indicate the direction of the ciliary currents in the grooves; the small arrows, that on the crests of the corrugations.) $\times 50$.

ABBREVIATIONS

c.s., cecal string; *excur. tubule*, excurrent tubule; *g.s.*, gizzard string; *inc. tubule*, incurrent tubule; *l.s.*, liver string; *s.*, sand; *t.i.*, impression of typhlosole in fecal pellet.

PLATE II



The excretory bodies and indigestible residues in the liver are voided periodically. These are passed simultaneously in minute mucous strings from all parts of the liver towards the central hepatic ducts, there converging into larger strings which pass in the direction of the hepatic vestibule. At the proximal end of the hepatic ducts this material fills most of the main duct. The combined currents in the grooves of the corrugations appear to exert a stronger force than those on the crests, so forcing the waste material directly into the hepatic vestibule (Fig. 11). There it is caught by the outward flowing ciliary currents on the major and minor pyloric folds and passed rapidly into the prointestine. The excretory bodies and indigestible residues passing from both lobes of the liver are compressed in the hepatic vestibule into one bulky string which is distinct from the cecal and from the gizzard string and may be called the *liver string* (Fig. 10). It is drawn out of the liver at the same rate as the cecal string passes out of the ceum. Both strings are usually found parallel to each other and uncoiled in the fecal pellets. The gizzard string, on the other hand, passes out much more slowly so that the cecal string occurs loosely and abundantly coiled therein (Fig. 10). A lapse of time seems to occur between the exit of the gizzard string and that of the liver string, as indicated by a conspicuous coiling of the cecal string between the last portion of the gizzard string and the forward end of the liver string. The gizzard string follows the liver string immediately, as indicated by no noticeable coiling of the cecal string between the two. There is also some evidence that, as the liver string is drawn from the liver, the pulsations of the stomach region cease. In animals opened for physiological observation of the tract, the stomach region was never pulsating when the liver strings were passing out of the liver. This is desirable to prevent the dismemberment of the strings and their mixing with food material brought into the liver by the pulsatory currents. The merger of the strings in the prointestine produces the fecal pellets.

The pylorus is composed of a complicated system of folds and passages, it is innervated by a pair of complex nerve plexuses and a nerve net, and all of the parts are exceptionally well vascularized. Functionally there is present in this portion of the tract an intricate system of counter ciliary currents and synchronized muscular movements, as well as partial vascular control of the folds. The pylorus is thus well equipped to convey digestive fluids from the liver to the gizzard and crop, to bear digested and semi-digested particles into the liver from the gizzard, to exclude large sand and other large particulate matter from the liver and transfer such residues to the prointestine, to receive waste material from the liver and transport it to the intestine, to act in conjunction with the cecum, liver, and hepatic ducts in shunting a continuous string of residual particles from the walls of these organs into the prointestine, to secrete fluids (of unknown nature) and finally to absorb fats and carbohydrates.

Liver

The liver is probably the most important organ of digestion in the alimentary system of the gastropods. Peczenik (1925) shows, as has been indicated in this work also in feeding experiments, that such proteins as egg albumen are engulfed and digested intracellularly in the digestive cells, and the indigestible residues are cast out in vacuoles. Krijgsman (1928) believes that digestive cells in *Lymnaea*

are also secretory as well as absorptive, as he has often observed numerous typical secretion granules in the liver cells of starved snails. Biochemical tests indicate that the greatest catheptic activity of the snail body is localized in the liver, yet none of this activity has been found in the fluid of the gut. This is in keeping with catheptic systems in other animals in which the enzyme has been shown to exist entirely as an intracellular protease. Hurst (1927) writes that in *Physa* fat and glycogen are stored in the digestive cells. Fat was also found in the lime cells of *Helix* by Grunbaum (1913). The problem of *what size* of food particle is engulfed through the distal membrane of the digestive cells is still an open question. It is likely, as indicated by the work of Krijgsman (1925, 1928) on *Helix*, that the lime cells function in storing and in periodically secreting a buffering agent which adjusts the pH of the gut juice; this point has not been investigated in *L. s. appressa*. The mucous cells of the liver provide the mucus utilized in the binding of the indigestible residues and the excretory bodies into the liver strings.

Amebocytes were found in varying numbers in the contents of the lumina of the liver, postesophagus, gizzard, and pylorus. These were similar to those seen in the blood. In some instances those in the gut contained fecal vacuoles so large as to force the cell into a peripheral lobate ring.

Rhythmic activity of the liver is suggested by inspection of sectioned liver tissue, of fecal pellets and of the living organ in various phases of its activity. Pulsatory movements of the stomach region are apparently interrupted only during the passage of liver strings and of gizzard strings. This may explain why smaller hepatic excretory bodies occur in the upper pylorus, gizzard, crop, and postesophagus in such insignificant numbers. If the pulsatory currents persisted during the elimination of the liver residues one would expect to find liver string detritus scattered over the gut in as great profusion as in the liver, along with the reddish colored secretions from the liver.

The inclusion bodies of the digestive cells of *L. s. appressa* have been studied in detail in the living cells of normally feeding snails, starved snails, snails fed on special diets and in preserved tissue sections. The egested bodies have been followed in the fecal pellets over a period of weeks. The results of the study clearly indicate the presence in the digestive cells of excretion bodies, of indigestible residues and of secretion in separate vacuoles.

Figure 8 illustrates a vacuole from the digestive cells which is filled with indigestible particles. These vacuoles measure 12 to 25 μ in diameter. In snails feeding on lettuce the contents are colored a greenish brown to dark brown and are composed of minute irregular particles, some of the larger ones of which measure about 3 μ in diameter. In the digestive cells they occur one per cell and in varying stages of particulate concentration. These constitute the bulk of the liver strings and retain their identity in fecal pellets which have been voided for several days.

The secretion granules are clearly evident in preserved histological sections stained with iron hematoxylin, especially grouped towards the distal area of the cell. Larger granules measure as much as 4 μ in diameter.

The excretion vacuoles (Figs. 4, 5, 6) when in the cells may measure as much as 25 μ in diameter, but in the fecal pellets have shrunk somewhat. In the living cells excretion bodies are found in variable form and color and are best observed when the cells are slowly pressed out under a cover slip as the fluids evaporate. The cell contents then pass rolling and turning from the ruptured cells, exposing the

different surfaces of the inclusions. There is one series in which the vacuoles range from small to large vacuoles containing variable numbers and sizes of minute blue-green, translucent, many-angled particles. The smaller particles are in constant Brownian movement, dancing around like a swarm of bees, and indicating the low viscosity of the fluids in the vacuoles (Fig. 4). In a second series the same variation in size of the vacuole is encountered but the blue-green bodies are present in groups of only one to four per vacuole and are spherical and smooth (Fig. 5). In a third series the vacuoles and bodies are identical in form to the second series, but the color of the bodies varies from a light brown to a dark solid brown. The largest of these bodies are sometimes found free of the vacuoles. When compressed under a cover slip they spread with a flowing viscous movement, much as a drop of heavy molasses spreads when pressed between two smooth surfaces. In the fecal pellets these vacuoles are usually found varying in diameter from 3 to 15 μ , and the vacuole membrane presses closely around the excretion body. A fourth type of excretion body is found which varies in diameter from 12 to 18 μ , is colored a dark brown with a smooth center and possesses a periphery of irregular markings, such that the body resembles a signet ring (Fig. 6). The excretion bodies described above are present principally in the liver strings, and only in negligible numbers in the cecal strings. The "browns" and "signets," particularly, stain with methylene blue and neutral red and do not dissolve in strong HCl. The different types described are not all present in any liver string in equal abundance at any one time, but vary independently, in a sequence which did not seem significant. Because of the transitional stages between some of these excretion bodies it is probable that they are all different phases of the same type of metabolic excretion; but the method of their formation is still a puzzle.

Intestine and rectum

Cilia on the typhlosole beat towards the lateral sides of the typhlosole (Fig. 3); those over the prointestine around the typhlosole beat circumferentially and somewhat obliquely from the dorsal to the ventral sides in a symmetrical pattern. The division of the currents occurs along the dorsal line of the prointestine. Over the pellet-compressor the cilia beat transversely across the intestine. The raphe bears a strong current which streams directly posteriad. Thus in the pellet-forming region, through ciliation and muscular movement, loose particles are gathered, rolled inward about the typhlosole and folded into a compact pellet. Strong ciliary currents in the remainder of the intestine and rectum are limited almost entirely to the costae, raphe, and pseudoraphe; cilia of the intercostal surfaces are relatively short and weak. Peristaltic activity is evident throughout the intestine and rectum, being noticeably strongest in the early portions of the prointestine, just behind the pellet-compressor.

Abundant vascularization of the prointestine, in contrast to the relatively poor vascularization of the esophagus, suggests that this region of the intestine may also function in the absorption of food and water.

Consolidation of the cecal and liver strings occurs at the hepatic vestibule; of the gizzard residues and cecal string, in the pellet-forming region. The cecal string as it is moulded in the cecum is already a smooth well cemented string and undergoes no further change as it is forced continuously across the outer margin of the atrium.

The liver string, characterized by a fine dark brown mottling and almost as well concentrated as the cecal string, receives a final transparent envelope of cementing fluid which binds the cecal string to it (Fig. 10).

The chief function of the pellet-forming region is that of consolidating and cementing the loose straggling gizzard residues which constitute by far the greatest bulk of the fecal pellet. The large numbers of mucous cells, basophilic flask cells and basal secreting cells about the pellet-forming region are indicative of the large quantities of cementing substance secreted during the moulding of the pellets. By means of ciliary streams and constriction of the tube at the pellet-forming region the gizzard residues are pressed into pellets, and the cecal strings, lying loosely

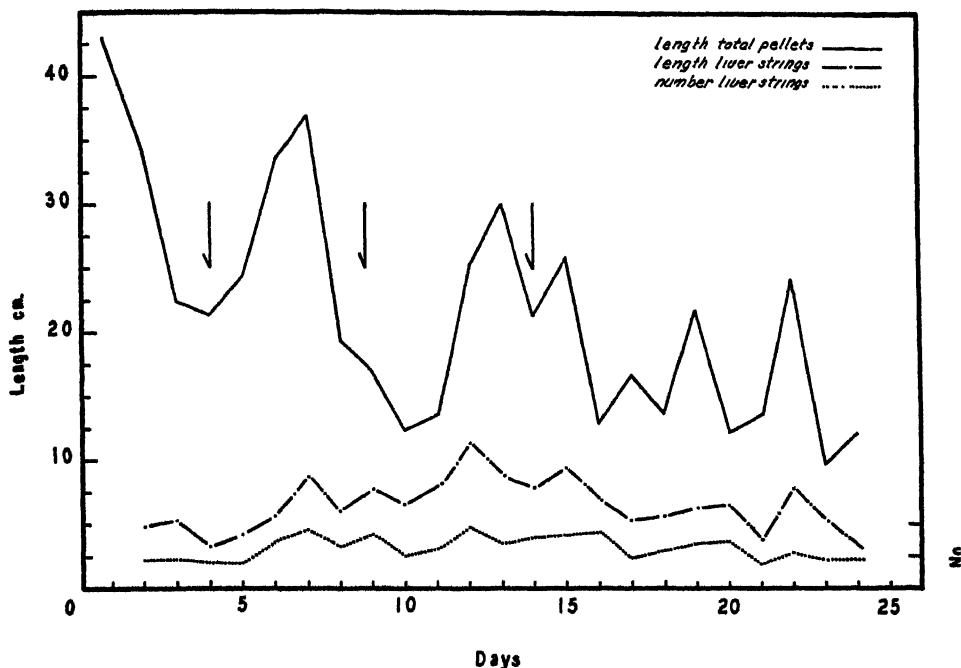


FIGURE 1. Length in millimeters of the liver and gizzard strings and number of liver strings of the fecal pellets, calculated on a twenty-four hour basis. These were voided in a period of twenty-four days by a forty millimeter *L. s. appressa*. The vertical arrows indicate the time at which egg masses were oviposited.

coiled in these residues, are simultaneously incorporated in the pellets. These are then forced out of the pellet-forming region by ciliary activity and by strong peristaltic movements which are noticeably stronger immediately behind the pellet-compressor. Peristaltic activity gradually diminishes in the direction of the anus. The conspicuous impression of the typhlosole remains in the fecal pellet, particularly in the gizzard string portion, as long after defecation as the pellet retains its form. Moore (1931) has found variable patterns in the fecal pellets of different Gastropoda and points out the importance of identification of animals by means of their pellets. A most striking fact about fecal pellet formation is the extreme com-

pleteness with which fecal material is compressed and cemented. This presumably prevents fouling of any portion of the tract.

For any given snail the diameter of the gizzard string portion of the fecal pellet is constant, varying principally with the size of the snail. The liver string varies in diameter from that of the gizzard string to that of the fecal string. Figure 1 indicates for a forty millimeter snail over a period of twenty-four days the rate and extent of voidance of fecal pellets. For the tabulation of this data the fecal pellets were collected daily and arranged end to end under the binoculars and measured to the nearest millimeter. The measurements given indicate only the lengths of the gizzard and liver strings, as the cecal string generally occurs embedded in the first two strings. The diameter of the gizzard string is reliably constant; that of the liver, less so.

Most conspicuous is the fact that the quantity of fecal pellets voided daily is quite variable from day to day. The quantity of gizzard strings fluctuates far more erratically than does that of the liver strings, indicating that the volume of material utilized by the liver is more constant than that which may pass through the gizzard. The number of liver strings is a more conservative indicator than the length of strings, and is probably not as accurate. Passage of food through the gizzard, and thus food consumption, seems to diminish during oviposition.

As indicated by the following data, feces were voided in about equal quantity day and night, with just a slight daily increase, over a period of twenty days (9 P.M. to 9 A.M., and 9 A.M. to 9 P.M., respectively) :

<u>Pellets</u>	<u>Night</u>	<u>Day</u>
Total length of pellets, mm.	1,987	2,134
Total length of liver strings, mm.	530	588
Total number of liver strings.	110	113

The total length of fecal pellets passed in the twenty-four days was 5,645 mm.; and the total length of liver strings, 1,491 mm., was passed in 289 liver strings, giving an average length of 5.1 mm. per liver string. Actually the liver strings varied in length from one to 10 mm. The average calculated length of fecal pellets passed in twenty-four hours was 235 mm.; of liver strings, 62 mm. In a normally feeding snail the sequence of the liver strings with the gizzard strings was always one of alternation. Liver strings do not generally mix with the gizzard strings. Gizzard strings as long as 52 mm. were found connecting liver strings. Three typical series of fecal pellets taken from days one, two, and three on Figure 1 are given below. The liver and gizzard strings are represented by the lengths in millimeters of the strings in the order of their elimination; the figures for lengths of the gizzard strings are italicized. The total time for elimination of the pellets is given to the right in parenthesis :

(1) 6 40 7 33 7 11 6 44 5	(5 hrs. 15 mins.)
(2) 48 7 52 8 13 4 50 5 38 6	(10 hrs. 15 mins.)
(3) 20 7 8 7 22 6 29 9 24 3	(10 hrs. 30 mins.)

As indicated by the curve for total fecal pellets in Figure 1 and by the lengths of the gizzard strings in the series above, consumption of food appeared to follow an alternating heavy and light cycle.

In snails deprived of food the elimination of the gizzard strings ceased and liver strings then became connected only by slender lengths of cecal strings. When starvation had continued for ten or more days nothing but delicate white cecal strings and a few much reduced liver strings containing metabolic excretion bodies were found in the intestine.

A. H. Rosenbloom (unpublished bachelor's thesis, 1942) by feeding colored food to *L. s. appressa* at different times through a period of a month found that in normally feeding snails of approximately forty millimeters shell length the minimum time for the passage of food from the mouth to the anus was two hours and twenty minutes; in snails previously starved for a week, five hours and fifty minutes. He found also that previously starved snails feed for a longer consecutive time than do normally feeding snails. The present investigation shows clearly that the alimentary system becomes completely emptied of food a few days after starvation commences. Considerably more food and a longer time are required for a starved animal to fill the alimentary tract with food to the point where fecal material is voided than for a normally feeding snail.

The rhythm of passage of liver strings is in keeping with the rhythm of the liver itself in which all digestive cells appear to assimilate food together and discharge indigestible residues simultaneously. This cycle, as indicated by the passage of liver strings, is not completely unvarying, because the number of liver strings discharged daily varied approximately from eight to nineteen. Thus the interval between the discharge of liver residues, probably the time during which the liver was digesting food, varied in this experiment from seventy-five minutes to three hours. It is possible that oviposition (Fig. 1) may account for some of the variability.

There seems to be nothing in the literature concerning fecal cycles in the Gastropoda. Some few scattered observations are reported on the length of the fecal pellets. For example, Heidermanns (1924) writes that a 48 mm. *L. stagnalis* with a 90 mm. intestine, eliminated 120 mm. of feces in 24 hours.

The long intestine is characteristic of the herbivorous snail nutrition of *L. s. appressa*. One of the most striking facts about the functioning of the alimentary system is the meticulous care with which all loose particles are collected and properly disposed of, in this way serving as a highly efficient sanitation system. The fecal pellets receive additional external layers of cementing material as they pass down the length of the intestine and rectum. The pH of the intestine is slightly more alkaline than that in the stomach region. As pointed out by Yonge (1935) mucus is an amphoteric protein whose viscosity is augmented by higher pH, thus more efficient consolidation of the feces occurs. Elimination of the fecal pellets through the anus is a fairly rapid and uniform process. The strong anal sphincter muscle remains tightly contracted except during defecation. Fecal pellets, being slightly heavier than water, settle slowly to the bottom of the aquaria. The marked efficiency of the mucoid coating over the feces is indicated by the extended period after defecation that pellets retain their identity. Thus it would seem that the alimentary system has not only become specialized in the maintenance of hygienic conditions within the system, but also in furthering a healthy external environment.

Fecal pellets are ingested by snails even in the presence of fresh food and the animals appear to derive some nourishment from them. It is to be recalled that the gizzard is not a thoroughly efficient grinding mechanism and in many cases,

particularly in the absence of sufficient fine sand, considerable unused available food passes out in the gizzard strings.

DISCUSSION

The question as to whether the radula slides over the cartilage independent of cartilage activity has been a favorite point of academic controversy with certain malacologists for some time (in Lymnaeidae see Geddes, 1879; Amaudrut, 1898; and Pelseener, 1935; in the Stenoglossa, a review: Carriker, 1943b). In *L. s. appressa* (and possibly in the majority of snails carefully investigated) there is no question but that the principal activity of the radula is that effected by the action of the cartilage and muscles under it, and a sliding of the total radula over the cartilage independent of the movement of the cartilage.

A study of the movements of the gut in *L. s. appressa* suggests that rather than the presence of different pH in the different portions of the gut, the pH may vary with the rhythms and secretions of the liver, the secretions of the salivary glands, the secretions of the unicellular glands of the gut wall and with feeding. It is quite unlikely that with the constant mixing of the gut contents as a result of the pulsatory movements at certain periods, the pH would vary markedly in the different lumina of the tract at any time. The wide range obtained between the maximum and the minimum pH's and the insignificant variation of the maximum and of the minimum pH's is in keeping with this suggestion. The partial isolation of the intestine from the movements of the stomach region is in keeping with the slightly higher pH found in the intestinal lumen.

The complexity and abundance of nervous tissues about the stomach region suggests a possible nervous control of the movements of the stomach region and of the liver. In its muscular structure there is no doubt that the buccal mass is the most complex organ in the alimentary system; functionally it appears that the region in and about the pylorus is the most intricate. The dense ramifications of blood vessels, the presence of two nerve plexuses, the intricate series of folds and the complicated ciliary streams in this region lend credence to this postulation.

Heidermanns (1924) has opened the question of the function of sand in the basommatophoran gizzard in his comparative study of *Ancylus*, *Planorbis*, *Physa*, *Lymnaea* and certain stylommatophorans. He points out that in land pulmonates the flaring portion of the esophagus is called the stomach, whereas in the aquatic pulmonates the esophagus is normal and the stomach has become differentiated into the crop, gizzard and pylorus. Thus the Stylommatophora have no organs that could properly be homologized with the stomach of the Basommatophora. The gizzard and, with few exceptions, sand in the tract are absent from the land pulmonates. The gizzard, he states, reaches its peak of specialization in *L. stagnalis* and probably rose by reason of the ingestion of sand with food. He observed that in all Basommatophora the gizzard originates in front of the first flexure of the gut, apparently as a muscular band whose primitive function was to dispose of sand masses tending to congest there. This primitive type of gizzard is exemplified by that of *Ancylus* and the intermediate type by that of *Planorbis*. Heidermanns in support of his theory of the origin of the gizzard through a specialization of a primordial portion of undifferentiated gut, attempted to show modification of the gizzard in one snail generation by the use of various diets. As might be anticipated, he got no significant structural changes.

The fact that *Lymnaea* possesses the gizzard grinding mill may explain the observation stressed by Heidermanns that the cellulase of this snail is less active than that of *Helix* which has not developed a gizzard and consequently needs a strong cellulase for the hydrolysis of the cell walls of plant food consumed.

There is striking similarity in the functioning of the alimentary tract of the herbivore *Onchidella celtica*, ably presented by Fretter (1943) in a recent paper, and that of *L. s. appressa*. Perhaps this similarity is not to be wondered at when, as Fretter writes, "Many of the features which the Onchidiidae share with the pulmonates may be attributed to the close origin of the two groups, the similarity of their diet and their air-breathing habit."

SUMMARY

1. A balanced physiological salt solution was developed which maintains contractions of the vas deferens for approximately 66 hours.
2. Cathepsin was found in greatest concentration in the liver and no activity could be ascertained in the gut fluids. Some trypsin was indicated in the salivary glands. Amylase showed greatest activity in the salivary glands and the liver.
3. Muscular activity of the alimentary system involves the manipulation of the mouth parts in the buccal mass, peristalsis in the remainder of the tract, marked pulsatory movements of the postesophagus, crop, pylorus and liver, and a kneading motion of the gizzard. The radula is moved principally by the action of the odontophore but also operates independently of it.
4. The entire alimentary system, with the exception of the gizzard and parts of the buccal cavity, is ciliated. The cilia show definite directional streams which function in propelling food particles, in sorting food and in consolidating fine refuse particles with the aid of mucoid substances.
5. Sand is consumed normally by the snail and is necessary for the proper functioning of the gizzard in the crushing of food particles. Very little trituration is performed by the mouth parts.
6. The pylorus is composed of a complicated system of folds and passages and counter ciliary currents and functions as a filter which permits only the soluble and the finer food particles to pass into the liver. It shunts the undigested residues from the gizzard into the prointestine.
7. In the liver the digestive cells function in secretion, assimilation, intracellular digestion and excretion. The indigestible foods and the excretory products, as variably shaped and colored inclusion bodies, are eliminated in vacuoles.
8. The cecum functions in collecting the finer residues from the liver and forces them in a continuous string into the prointestine.
9. The residual material coming from the gizzard, liver and cecum is characteristic for each organ and is readily identified as distinct in the fecal pellet.
10. The prointestine is specialized in the final consolidation of gizzard, liver and cecal strings with the aid of cementing substances secreted by the basophilic flask cells and the basal cells.
11. The rhythmic nature of the liver is disclosed, principally by a study of the fecal pellets.
12. *L. s. appressa* is an herbivore. Food bits are cut away by the radula and swallowed. In the buccal cavity the food receives mucus from the buccal gland

cells, mucous cells and the salivaries and enzymes from the latter. Temporary storage and initial digestion occur in the postesophagus. Digestive fluids pass up from the liver in the pulsatory movements of the stomach region which keep the fluid gut contents in constant circulation. The crop, gizzard and anterior portion of the retrocurrent passage of the pylorus comminute the food. Amebocytes present in the gut contents appear to aid in digestion. Soluble and fine particles of food pass through the pyloric filter into the liver where it is assimilated by the digestive cells. Assimilation also occurs in the pylorus and absorption possibly in the intestine. There is some evidence that the pulsatory movements of the stomach region cease during the passage of the gizzard and the liver strings.

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TEMPORARY PAIR FORMATION IN *PARAMECIUM BURSARIA*¹

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In *Paramecium bursaria*, the two members of a conjugating pair normally remain united twenty to thirty-eight or more hours. During this time various nuclear processes take place, including three pregamic divisions, exchange and fusion of pronuclei, and three post-zygotic divisions. Clones that are capable of undergoing normal conjugation as described above are called "normal clones."

But there are some clones of this species which are abnormal² in that when they are mixed with normal clones the pairs formed are not lasting but separate within a few hours. An examination was made of such temporary pairs in order to discover what nuclear or other changes occur in them.

Fourteen clones of *Paramecium bursaria*, all belonging to Variety I, have been used in the present study. These clones, all of which were collected in nature, are listed in Table I with data on each clone including (1) the mating type to which it belongs and (2) the locality where it was collected.

TABLE I

Clones of Paramecium bursaria employed in study of temporary pair formation

Clone number	Original designation of clone	Mating type	Locality collected
1	SAa5	A	Santa Ana River, Cal.
2	Or3	A	Vicinity of Capistrano, Cal.
3	SGa3	A	San Gabriel River, Cal.
4	BG35	A	Los Angeles, Cal.
5	La3	B	Laguna Canyon, Cal.
6	SAa7	B	Santa Ana River, Cal.
7	UC13	B	Los Angeles, Cal.
8	BH2	B	Beverly Hills, Cal.
9	SAa4	D	Santa Ana River, Cal.
10	LP10	D	Lone Pine, Cal.
11	UC14	D	Los Angeles, Cal.
12	SAa1	C	Santa Ana River, Cal.
13	BH7	C	Beverly Hills, Cal.
14	BH101	C	Beverly Hills, Cal.

Clones 1-11 are normal clones in that they are capable of undergoing normal conjugation. Clones 12-14 are abnormal clones in that when they are mixed with normal clones the pairs formed are not lasting but separate within a few hours.

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² These clones are considered abnormal here only because they are incapable of taking part in the formation of lasting pairs when they are mixed with other, normal clones.

The animals were cultured in essentially the manner described by Jennings (1939). For cytological study the animals were fixed in Schaudinn's fluid containing glacial acetic acid, stained in iron hematoxylin, and destained in saturated aqueous solution of picric acid, following the technique the writer has described (Chen, 1944).

EXPERIMENTAL STUDIES

Most of the present work was done with the two abnormal clones (BH7, SAA1), although some study was also made on a third abnormal clone (BH101). All of these three abnormal clones belong to mating type C of Variety I. These abnormal clones were mixed with normal clones. Eleven such normal clones (all belonging to Variety I) were used. Four of these normal clones belong to mating type A; four to type B; and three to type D (see Table I).

As an example of the phenomenon of temporary pair formation, the reaction between the abnormal clone SAA1 and the normal clone UC13 will be described. On November 27, 1940, a large number of animals belonging to each of these two clones were mixed at about eleven o'clock in the morning. Strong agglutinative mating reaction occurred almost immediately. Half an hour after mixture, pairs were being formed. An hour after mixture (about noon) many pairs were formed. But in the early afternoon the pairs broke apart into single animals. By five o'clock all but a few pairs had separated. By evening all had separated.

Such temporary pair formation was also observed when the abnormal clone SAA1 was mixed with the following normal clones: Or3, SGA3, SAA5, La3, SAA7, BH2, SAA4, LP10, and UC14; or when the abnormal clone BH7 was mixed with the normal clone LP10; or when the abnormal clone BH101 was mixed with the normal clone BG35.

If such a mixture was placed in a moist chamber and kept from drying (with occasional replacement of the fluid that evaporated), the typical agglutinative mating reaction and temporary pair formation recurred the following day and almost daily over a period of many days. Some such mixtures were kept under daily observation over a period of nineteen days. The following is the characteristic daily behavior of the animals in such a mixture. The agglutinative mating reaction occurs in the late morning. By noon, many pairs are formed. These pairs persist for a few hours. Between four and six o'clock in the afternoon only a few pairs are found. In the early evening one or two pairs may remain; none can be found after nine o'clock in the evening.

CYTOLOGICAL STUDIES

Nuclear conditions in the clones that form only temporary pairs

The writer has made a cytological study of twenty-one abnormal clones, including the two clones BH7 and SAA1, and nineteen of the twenty-two such clones described by Jennings (1944). It was found that fifteen of these clones possess micronuclei, while six appear to be amiconucleate.

Thus the amiconucleate condition is not the general cause of the peculiar behavior of these abnormal clones. It is probable that the persistence of the amiconucleate condition is a *result* of the inability to conjugate and acquire a micronucleus, rather than the cause of it. Apparently there are conjugating and non-conjugating

ances of amiconucleate ciliates. In nature those that can conjugate do so and acquire a micronucleus, leaving in the amiconucleate condition only those incapable of conjugation. In my experience with *P. bursaria*, which includes a study of the nuclei and chromosomes of many clones (collected from different parts of the United States, Canada, Russia, England, Ireland, and Czechoslovakia), the only amiconucleate animals found in nature are those which cannot conjugate. Since they cannot conjugate, it is likely that such clones will be permanently amiconucleate. In nature any amiconucleate animal that can conjugate would not remain amiconucleate for long, since it would become micronucleate after mating with a normal animal from whom it receives a pronucleus as a result of conjugation (Chen, 1940).

Amiconucleate animals that can conjugate have been found in *P. bursaria* (Chen, 1940)³ and in *Euplotes patella* (Kimball, 1941). They arose spontaneously in laboratory cultures.

Nuclear changes in temporary pair formation

To determine whether nuclear changes occur in temporary pair formation, a series of preparations were made, in December, 1940, of temporary pairs (abnormal clone SAa1 \times normal clone UC13)⁴ and a number of separated animals belonging to the latter clone. The material included pairs 5 to 6 hours after onset of temporary mating, separated animals a few hours after separation, 13 to 17 hours after separation, and 21 hours after separation. The micronuclei in these temporary pairs and separated animals were compared with the micronuclei of vegetative animals of clone UC13 (not mixed with any other clone). It was found that micronuclei in the majority of the temporary pairs and of the separated animals were slightly swollen. In some, no nuclear changes were apparent.

In June, 1943, a series of preparations were made of temporary pairs (abnormal clone BH101 \times normal clone BG35)⁵ and a number of separated animals belonging

³ The writer has recently found some additional cases of conjugation between amiconucleate and normal animals in *Paramecium bursaria*, in Variety III. (Normal nuclear changes occur in the conjugants having the micronuclei.) These amiconucleate animals arose spontaneously in laboratory cultures.

Schwartz (1939) in a brief preliminary paper reported "conjugation" in *Paramecium bursaria* between amiconucleate and normal animals and between two amiconucleate animals. In view of the lack of details in this report, it is impossible to tell whether temporary or lasting pair formation took place.

⁴ Clone SAa1 appears to be amiconucleate; clone UC13 has a deeply staining micronucleus.

⁵ Clone BH101 has a small, lightly staining micronucleus; clone BG35 has a relatively large, deeply staining micronucleus.

EXPLANATION OF FIGURES

FIGURES 1-34. Micronuclei of animals belonging to clone UC13 before, during, and after temporary pairing with animals of clone BH7 (drawn by Mr. Earl Nielsen). All drawings were made with a camera lucida. $\times 3,300$.

FIGURES 1-5. Resting micronuclei of vegetative animals.

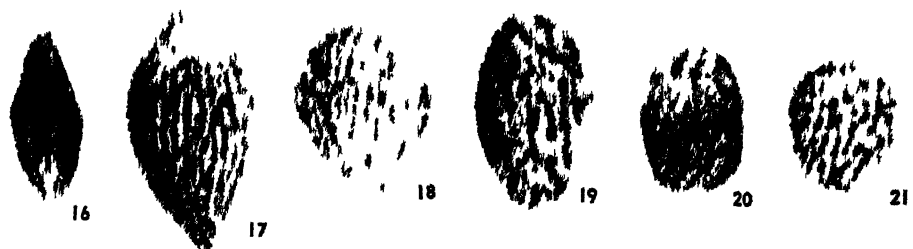
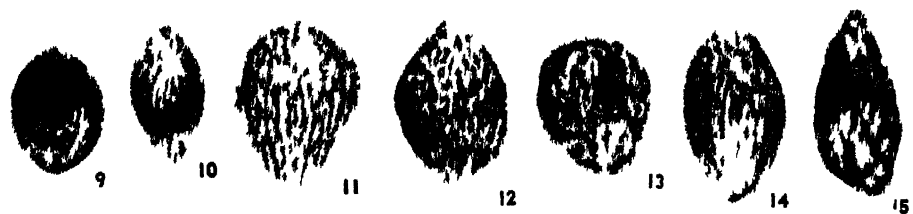
FIGURES 6-10. Micronuclei in the members of temporary pairs 4 hours after onset of pairing.

FIGURES 11-16. Micronuclei in the separated animals 18 hours after separation.

FIGURES 17-22. Micronuclei in the separated animals 30 hours after separation.

FIGURES 23-28. Micronuclei in the separated animals 42 hours after separation.

FIGURES 29-34. Micronuclei in the separated animals 51 hours after separation.



FIGURES 1-34.

to the latter clone. The material included pairs 3 to 4 hours after onset of temporary pairing, and separated animals 2 hours after separation, and a day after separation. It was found that the micronuclei in the majority or most of the temporary pairs and separated animals were slightly swollen. In others no nuclear changes were apparent.

In October, 1944, a series of preparations were made of the temporary pairs (abnormal clone BH7 \times normal clone UC13)^{*} and separated animals belonging to the latter clone. The material included pairs 4 hours after onset of temporary mating, and separated animals 7 hours after separation, 18 hours after separation, 30 hours after separation, 42 hours after separation, 51 hours after separation. A series of preparations of vegetative animals of clone UC13 (not mixed with any other clone) were used as controls (Figs. 1-5). It was found that the micronuclei in nearly all of the temporary pairs and separated animals were slightly but noticeably swollen (Figs. 6-34). This is true even of the separated animals 51 hours after separation (Figs. 29-34), indicating that the physiological effect of the contact between the animals in temporary pairing (as shown by the swelling of the micronucleus) is of long duration.

GENERAL RELATIONS

The temporary pair formation described in the present paper is similar to that reported by Sonneborn (1942) in *P. aurelia* and by Jennings (1944) in *P. bursaria*. Sonneborn (1942) concluded from his data that cell adhesion occurring in the initial stage of the mating reaction and cell fusion occurring during subsequent conjugation are due to two different mechanisms.

SUMMARY AND CONCLUSIONS

1. In normal conjugation of *Paramecium bursaria*, the two members of each pair remain united for 20 to 38 or more hours, during which time various nuclear processes take place including three pregamic divisions, exchange and fusion of pronuclei, and three post-zygotic divisions. Clones that are capable of undergoing normal conjugation as described above are called "normal clones."
2. Some clones of this species are abnormal in that when they are mixed with normal clones the pairs formed are not lasting but separate within a few hours.
3. In temporary pair formation, the animals of diverse mating types when mixed exhibit the typical agglutinative mating reaction. Within an hour many pairs are formed but in a few hours these pairs break apart into single animals.
4. If such a mixture is placed in a moist chamber and kept from drying (with occasional replacement of fluid that evaporates) such agglutinative mating reaction and temporary pair formation will recur daily over a period of many days.
5. Cytological study of 21 such abnormal clones shows that most of these clones have micronuclei; some appear to be amiconucleate. Therefore, amiconucleate condition cannot explain the incapacity for taking part in the formation of lasting pairs. It is probable that the persistence of the amiconucleate condition is a *result* of the inability to conjugate and acquire a micronucleus rather than the cause of it.
6. In temporary pair formation, there are no conspicuous nuclear changes either in the pairs or in the animals after their separation. In the majority of the tempo-

^{*} Clone BH7 appears to be amiconucleate.

rary pairs and separated animals, there is, however, a slight swelling of the micronuclei. This swelling persists for a considerable length of time after the separation of the animals, indicating that the physiological effect of the contact between the animals in temporary mating (as shown by the swelling of the micronuclei) is of long duration.

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THE SPACE-TIME PATTERN OF SEGMENT FORMATION IN ARTEMIA SALINA

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INTRODUCTION

The present work was carried out in an attempt to arrive at a primary understanding of the regularities and laws in the phenomenon of metamerism segmentation, as related to the shape and size of animals. To date this phenomenon, although of widespread occurrence amongst the higher animal phyla and thus probably an integral part in the more complex patterns of evolutionary organization, was nevertheless surprisingly rarely, if at all, subjected to analytical inquiry. The reason for this can probably be found in an essential lack in the past of well-defined concepts about the interrelations between mass, shape, growth, and degree of development of living organisms. The problem of segment formation in relation to size and shape is primarily one involving a clear appreciation of the dynamic geometry of living matter, and initial insight into the problem can therefore only emerge from rigorous observation on a quantitative level, followed preferably by geometrical and mathematical analysis. Such a method has been employed in the present work, and the results gained are conclusive enough not only to point the way for further study of the problem at hand, but also to promise reasonable success in the application of the quantitative, geometrical method to questions of biological space-time pattern in general.

The choice of *Artemia* has proven particularly fortunate for a study of metamerism segmentation. The animal, held to be amongst the most primitive of living Crustaceans (Lockhead, 1941), develops few, if any, specialized structural features which would ordinarily tend to obscure the fundamental processes of morphogenesis. Moreover, the development of as many as nineteen body segments, a further primitive trait, is of obvious advantage in the investigation of the underlying principles of formation. Also, *Artemia* is easily obtained and can be reared in the laboratory without difficulty.

METHODS AND MATERIALS

Larvae of *Artemia salina* were obtained from commercial, air-dried egg cysts. Since excystment is retarded or inhibited in water above a certain salinity (Jennings and Whitaker, 1941), water of a specific gravity of 1.020 was used throughout as the initial medium. The egg shells cracked open usually 12 to 18 hours after contact with the water, and emergence of the larvae (Whitaker, 1940) took place

between 18 and 24 hours. Portions of five stock solutions of brines with different salt concentration were employed as further media. The solutions were obtained from the original sea water by either diluting with doubly glass distilled water or concentrating with NaCl to specific gravities of 1.022, 1.033, 1.047, 1.066, and 1.085, respectively. All solutions were vigorously aerated daily, and possible deviations from the proper specific gravity were adjusted in weekly intervals. All work was carried out at room temperature, corresponding to an average water temperature of 21–22° C. As soon as the embryos had emerged, still enclosed within the fine hatching membrane, they were transferred to water from either of the five stock solutions. The moment of hatching, occurring within 24 to 30 hours after the cysts had first made water contact, was taken as zero time for all further determinations.

For observation the larvae were reared singly, in heavy crystal watch glasses. In the course of several observational series, a total of up to 100 individuals were observed, at least for certain periods of their development; of the 100, about 25 individuals, evenly distributed among the solutions, were reared from hatching to the adult stage. The presence or absence of a molted shell, the time, the temperature, the stage of development reached, and a series of measurements on bodily proportion were recorded for each individual twice daily in the earlier stages and daily for later stages. The animals were fed once every two days on a sea-water-yeast suspension. Each watch glass containing an animal was covered so that evaporation was nearly abolished, but a minimum of air circulation was always allowed for to equilibrate the CO₂ released by the yeast and the animal. The water was changed at two-day intervals for the younger stages and daily for older ones.

Larval body measurements were taken under the microscope with the help of a hemocytometer slide whose grid allows the direct reading off of lengths of 50 micra and consistent estimations of lengths of 10, 20, and 30 micra. If the larva is placed on a coverslip with a minimum of water, the whole can be adjusted in relation to the grid; evaporation is sufficiently slow to allow five or six measurements at a time. The error inherent in this method, viz., the parallax due to the thickness of the coverslip, is small enough to be negligible; also, since all measurements were taken in this way, the relative values are consistent.

PRELIMINARY OBSERVATIONS

Barigozzi (1939) and Rugli (1941) observed the total developmental time from hatching to the adult stage of *Artemia* to be 3 to 4 weeks. This is true as a broad generalization, but with the egg cysts in the various salinity media here employed, certain statistically preferred tendencies become apparent, expressed empirically by

$$D_x = D_0 \cdot S_x^{-6.8}$$

where D_x is the time, in days, for complete development from hatching in a solution of salt concentration x ; D_0 , the (hypothetical) time, similarly, for development in distilled water (the algebraic value turned out to be 36.55); and S_x , the specific gravity of the solution of concentration x . This relation holds good only in a statistical sense, within a specific gravity range of 1.020 and 1.1; it indicates that with higher salt concentrations the rate of development tends to be greater (Fig. 1). Irrespective of concentration a sigmoid curve of growth is always obtained.

Morphologically, different salinities have no differential effect on relative body proportions, a result to be expected in view of the conclusions of Bond (1932). An inverse relation between total size and salinity, observed by Bond, Heath (1924), and Warren (1938) for larvae from non-excysted eggs in the natural habitat, however, could not be observed for the excysted larvae here used; in the latter, total sizes are identical at equivalent stages of development, irrespective of salinity.

The number of molts between hatching and sexual maturity is not constant. Even when reared in the same medium, slight differences in molting frequency between several larvae may occur. Moreover, there exists a rough statistical relation between salinity and the total number of molts, approximating closely the

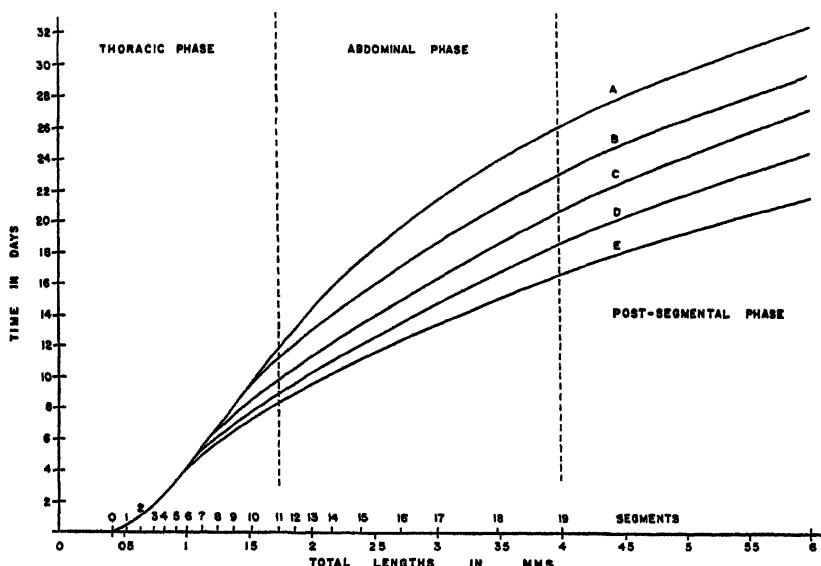


FIGURE 1. The effect of salinity on developmental time, from hatching to sexual maturity; absolute growth curves. Abscissa: total larval length; Ordinate: time in days. A-E, media of salt water, specific gravities from 1.022-1.085 respectively; numbers 1-19 above abscissa refer to number of body segments present.

above relation between salinity and the time required for complete development; in general, however, the number of molts for a given salinity is somewhat lower than the number of days required for development. In larvae from excysted eggs and under artificial food conditions, a range of 12 to 16 molts was observed between hatching and maturity, at a specific gravity of 1.085; this compares with 25 to 29 molts at a specific gravity of 1.022, and gradually decreasing molting frequencies for the intermediate salinity ranges. A staging of larval development according to molts, as Heath has done for non-excysted individuals, would therefore not be possible in the present case. Heath's 13 stages would hold for excysted larvae only when reared in brine of a specific gravity of 1.085; even then certain definite differences in the degree of development of equivalent molting stages can be

observed, as comparison of Heath's descriptions with those below makes apparent.

With increasing developmental age the duration of instars increases; a 12 to 24 hour interval between molts in the younger stages compares with 24 to 30 hour intervals in older ones. The two factors of salinity and developmental age also determine the size increase between molts; for higher salinities, as well as for older larvae, the size increase is greater. There is no observable relation however between the time at which a molt occurs and the size or the developmental stage attained, irrespective of whether test larvae are reared in the same or at different salinities. Molting is also greatly influenced by the food supply. Starving animals do not molt; after 3-5 days an abortive attempt at molting is made which usually results in the death of the animal. Conversely, overfed larvae may molt twice in rapid succession without undue increase in size.

ANALYSIS OF SEGMENT FORMATION

Observations and definitions

The larval development of *Artemia* can best be dealt with in terms of the number of body segments present. The first three segments become visible almost simultaneously at a total larval size of 0.745 mm. (stage 3), after the embryonic yolk has been digested away, and the termination of the hatching, nauplius, and metanauplius stages can therefore be represented as the termination of stages 0, 1, and 2, respectively; at the end of any following stage the stage number will thus indicate directly the number of body segments present. It will be convenient to distinguish between a thoracic period of development, comprising the first 11 stages, and an abdominal period, including stages 12 to 19; the latter can again be divided into a genital period (stages 12 and 13) and a post-abdominal one (stages 14 to 19).

Except for the first three, each individual segment is initially recognizable as a transverse ring of thickened mesoderm, the segment rudiment, immediately underneath the otherwise smooth epidermal layers (segmental stage *a*). Later, partial transverse constrictions appear externally in the epidermis and the chitin, in a plane just posterior to that of the segment rudiment (segmental stage *b*). Eventually, the constrictions become complete and deepen, with a concomitant bulging out of the body wall in the region of the segment rudiment (segmental stage *c*). At this stage, the segment can be considered "laid down," its shape resembling more or less a short cylinder. In thoracic segments, appendage buds appear in stage *c* ventro-laterally, on either side. The segments are considered mature when their pairs of swimming appendages first become independently motile. Stages *a* to *c* of the first and second, and stages *a* and *b* of the third segment can never be clearly seen; the first stages of these segments are attained prior to hatching and during the nauplius and metanauplius phases, when the presence of dense yolk conceals details of structure. As these segments become plainly visible in the third stage of the thoracic period, segment 3 is in stage *c*, but segments 1 and 2 are already correspondingly ahead, both in size and the degree of their development.

At the end of the thoracic period the 11th segment has reached stage *c* and the first five segments have become mature. The 11th segment attains maturity at the end of the abdominal phase of development (stage 19). Appendage buds similar to those on more anterior segments also develop on segments 12 and 13. But instead of developing into swimming appendages the buds on either side of

both segments enlarge, and in the female fuse into a sac in stage 18, forming the left and right brood pouch; no male larvae were investigated. The remaining six segments develop similarly from segment rudiments, but no appendage buds are ever formed and stage *c* represents the first stage of maturity. At the end of the abdominal period the 19th and last segment has become mature. In the head, the ocellus becomes pigmented in stage 2 and the compound eyes in stage 4. The maxillae and maxillulae also form in stage 2. The end of stage 19 marks the time when the gnathobase and the setae have been lost entirely from the second antenna. After stage 19 an arbitrary number of non-segmental stages ensue before sexual maturity is reached.

When individuals in identical stages of development from the same or from different salinity media are compared, it is strikingly apparent that total lengths and body proportions in general fall within well-defined size-classes; the deviations from the underlying averages in no case exceed ± 3 per cent. In Table I the averages of a variety of body measurements are shown, from the 25 individuals watched throughout development, with the stage number as the basis of calculation; these values, within ± 3 per cent, are true for individuals from any of the salinities here examined. A schematic diagram of an *Artemia* larva indicates, in Figure 2, how the various entities have been defined. Head length is understood to include maxillar and maxillular segments. The length of a segment refers to axial and the width to its lateral extent. Total abdominal length is the length of the segmental portion, whether actually cut up into segments or not, plus the length of a terminating anal piece; the segmental portion is the pygidium of annelid forms, and in *Artemia* is readily distinguished from the anal piece, or urosome, by a constriction. During the abdominal period of development, the segmental abdomen contains a genital region composed of segments 12 and 13, as well as a post-abdomen (presumptive segments 14 to 19) with segmented and non-segmented portions.

From observation and from examination of the data in Table I the following facts concerning the formation of segments in relation to larval shape and size are consistently found to occur:

1. Every thoracic segment when newly formed (stage *c*) has a fixed length of 0.03 mm. and a fixed width of 0.144 mm.
2. Every time a new thoracic segment is laid down in stage *c*, preceding segments increase in length and in width.
3. Throughout the thoracic period, the segmental part of the abdomen has a constant average length of 0.249 mm.; its anterior width, being slightly smaller than the width of the newest segment, is also constant ($C = 0.142$ mm.).
4. During the thoracic period, the lateral contour-lines of the thorax are straight lines converging posteriorly; the lateral abdominal contour-lines are also straight, but generally they converge with a greater degree of taper than the thoracic contours.
5. Appendages are longer the more anterior they are; the line joining the tips of the appendages on one side of the body is more or less a straight line.
6. As the 11th segment appears in stage *c*, the 5th segment has matured and the 19th segment has appeared in stage *a*.
7. Between stage *a* and stage *c* of the thoracic segments, 4 stages of larval development intervene; an interval of more than 4 developmental stages is necessary for an abdominal segment to reach stage *c* from stage *a*.

TABLE I
Segmental development and larval body proportions, in millimeters

Stage Number	No. SR	No. S	SM	Tot. L	HL	T	TAL	A	GL	P	SPAL	U	TS	TSL	PS	W	WTSL	WA	WASL	WU
0	4	0		0.425	0.206		0.219										(0.142)			
1	5	1		0.524	0.209	0.030	0.285	0.215				0.070	0.030	0.030		0.144	0.144			0.080
2	6	2		0.637	0.249	0.070	0.318	0.238				0.080	0.040	0.030		0.146	0.145			0.080
3	7	3		0.745	0.289	0.122	0.334	0.241				0.093	0.050	0.030		0.150	0.136			0.085
4	8	4		0.841	0.313	0.185	0.343	0.243				0.100	0.058	0.031		0.155	0.139			0.097
5	9	5		0.934	0.339	0.242	0.353	0.244				0.109	0.065	0.028		0.162	0.138			0.105
6	10	6		1.025	0.343	0.311	0.371	0.255				0.116	0.076	0.033		0.167	0.139			0.108
7	11	7	1	1.125	0.366	0.390	0.369	0.237				0.132	0.086	0.029		0.179	0.143			0.107
8	13	8	2	1.236	0.382	0.480	0.379	0.243				0.136	0.097	0.030		0.191	0.144			0.111
9	15	9	3	1.386	0.400	0.600	0.386	0.240				0.146	0.110	0.031		0.209	0.143			0.118
10	17	10	4	1.540	0.407	0.733	0.400	0.246				0.154	0.120	0.033		0.225	0.150			0.120
11	19	11	5	1.716	0.429	0.861	0.426	0.246				0.180	0.127	0.027		0.245	0.150			0.125
12		12		1.855	0.455	0.87	0.53		0.04	0.290		0.200	0.135	0.035		0.25		0.150	0.145	0.13
13		13		2.000	0.460	0.87	0.65		0.10	0.325		0.225	0.130	0.035		0.25		0.160	0.145	0.13
14		14	6, 14	2.155	0.475	0.93	0.75		0.12	0.38	0.06	0.25	0.135	0.045	0.06	0.25		0.170	0.148	0.13
15		15	7, 15	2.400	0.520	1.05	0.83		0.14	0.45	0.16	0.24	0.145	0.050	0.08	0.35		0.185	0.150	0.13
16		16	8, 16	2.720	0.550	1.20	0.97		0.165	0.58	0.27	0.25	0.150	0.060	0.09	0.33		0.190	0.140	0.14
17		17	9, 17	3.050	0.570	1.35	1.13		0.185	0.73	0.48	0.25	0.175	0.065	0.12	0.33		0.200	0.137	0.14
18		18	10, 18																	
		12, 13	3.450	0.600	1.50	1.35			0.19	0.91	0.75	0.25	0.175	0.075	0.15	0.35		0.210	0.139	0.14
19		11, 19	4.000	0.650	1.70	1.65			0.23	1.13	1.08	0.27	0.180	0.095	0.18	0.37		0.230	0.144	0.14

Legend: No. SR, number of segment rudiments; No. S, number of segments present; SM, segments matured in this stage; Tot. L, total larval length; HL, head length; T, thoracic length; TAL, total abdominal length; A, length of segmental abdomen; GL, genital length; P, post-abdominal length; SPAL, length of segmented post-abdomen; U, urosomal length; TS, length of first thoracic segment; TSL, length of last thoracic segment; PS, length of a post-abdominal segment; W, width of first thoracic segment; WTSL, width of last thoracic segment; WA, width of first abdominal segment; WASL, width of last abdominal segment; WU, anterior urosomal width.

8. Between stage *c* and maturity in the first 5 segments, 6 stages of development intervene; 8 stages intervene before maturity of segments 6 to 11.

9. The anterior width of the non-segmented portion of the abdomen during the abdominal phase is of a fixed and constant magnitude and identical to the constant anterior width of the abdomen during the thoracic phase; thus at the end of stage

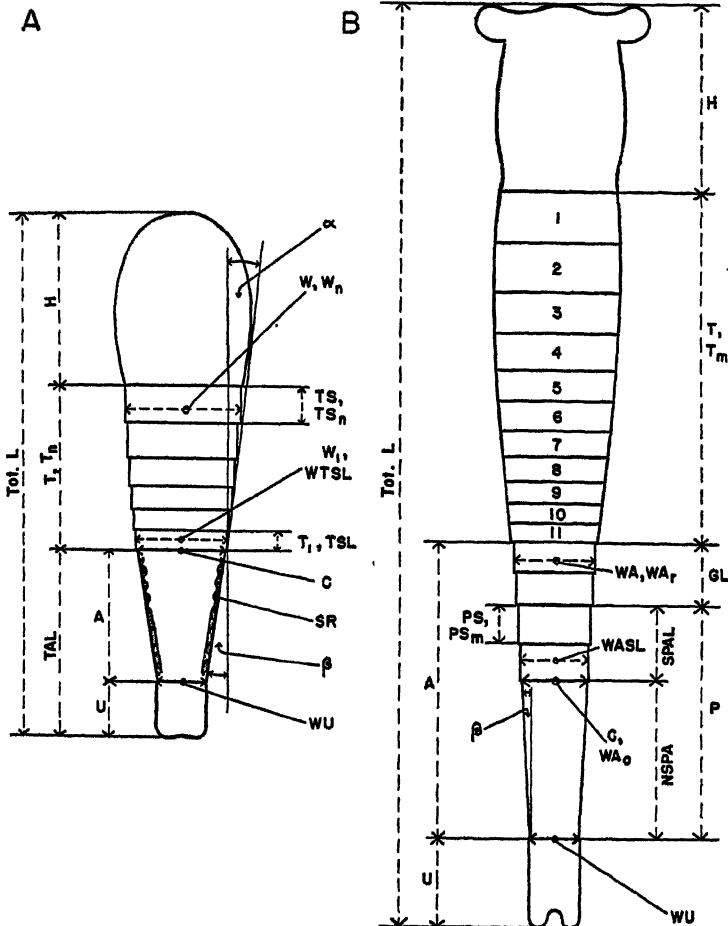


FIGURE 2. Schematic diagram of a larva of *Artemia salina*. A, larva in the thoracic period; B, larva in the post-abdominal period of development. *A*, length of segmental abdomen (pygidium); α , angle of thoracic taper; β , angle of abdominal taper; *C*, W_o , WA_o , constant anterior width of segmental abdomen; *GL*, length of genital region; *H*, head length; *NSPA*, non-segmented post-abdomen; *P*, length of post-abdomen; *PS*, *PS_m*, length of a post-abdominal segment; *SPAL*, length of segmented post-abdomen; *SR*, segment rudiment; *T*, *T_n*, *T_m*, length of thorax; *TAL*, total abdominal length; *TS*, *TS_n*, length of first thoracic segment; *TSL*, *T₁*, length of last (newest) thoracic segment; *Tot.L*, total larval length; *U*, urosomal length; *W*, W_n , width of first thoracic segment; *WTSL*, W_1 , width of last thoracic segment; *WA*, WA_r , width of first abdominal (12th) segment; *WASL*, width of last (newest) abdominal segment; *WU*, anterior width of urosome.

19, the width of the urosome is the same as the posterior width of the head in stage 0 when segmental development started. A constant width has seemingly travelled down the larva.

10. At the beginning of stage 12, the segmental abdomen starts to grow in length at a fast rate, having retained a constant length in the thoracic period.

11. Segments 12 and 13 are of equal length at any time after their formation; they are individually always somewhat longer than the 11th thoracic segment and become progressively shorter, relatively, than the 14th segment. At stage 18, 6 developmental stages after segment 12 has reached stage *c*, segment 12 and 13 fuse to form the brood pouch in the female and can then be considered matured. The interval for attainment of maturity is thus equal to the similar interval in the first 5 thoracic segments.

12. Segments 14 to 19 are not of equal length when formed; more posterior segments, when formed, are longer than more anterior ones when formed. Also, any one of these segments has always one-sixth of the length of the post abdomen, and at a given time post-abdominal segments are of equal length.

13. When segment 19 reaches stage *c*, the 11th segment attains maturity.

14. During the abdominal phase, the thorax changes shape in the following way: the 2nd segment becomes longer and wider than the 1st, then the 3rd larger than the 2nd, etc., and the 5th becomes the largest, coincident with the end of stage 19. As a result, the lateral thoracic contours become curved, the widest part of the thorax being at segment 2 in stage 16, at segment 3 in stage 17, etc., and at segment 5 in stage 19.

15. Similar differential increases take place in the appendages; at stage 19, the 5th pair of swimming appendages is longest and appendageal length regularly decreases towards the 1st and the 11th pair. The line joining the appendage tips on one side of the thorax is now also curved.

16. During the abdominal phase, and paralleling the differential increases in the thoracic segments, a progressive dorsal thoracic curvature develops, with an analogous shift backwards of the maximal flexure; the latter arrives similarly at segment 5 at the end of stage 19. Due to this flexure the head now appears bent ventrad.

17. The lateral contours of the abdomen remain straight lines throughout the abdominal phase, with a definite taper directed backwards.

18. At the end of stage 19 segmental development is completed; further development is still to take place in the head. The essential overall shape of the animal as now established, i.e., the possession of a barrel-shaped thorax and a straight tapering abdomen, is carried through to sexual maturity, although changes of detail do still occur.

These observations are now to be interpreted and integrated analytically.

The thoracic phase of development

The lengths of the first thoracic segment, in successive stages, are 0.03, 0.04, 0.05, 0.058, 0.065, 0.076 mm., etc. (Table I). The differences between these values, taken for all 11 thoracic stages, are very close to an average difference of 0.0097 mm. The length of the first thoracic segment in successive stages can

therefore be expressed as an arithmetical series

$$TS_n = (TS_1 - TS_0) + (n - 1) \cdot \Delta s \quad (1)$$

where TS_n refers to the length of the first thoracic segment at stage n ; n , to the successive stage numbers from 1 to 11 (and thus to the number of segments present at the time); $(TS_1 - TS_0)$, to the initial length of the first segment at the end of stage 1; and Δs , to the increase of segmental length per stage (0.0097 mm.). The expression would mean that the first segment grows in length by a constant amount Δs during each stage.

Since every other thoracic segment is known to start off with an identical value for $(TS_1 - TS_0)$, viz., 0.03 mm., it could be possible that other thoracic segments also increase a constant amount Δs during each stage. If that were true, then the newest segment, at any given stage, would have a length of $(TS_1 - TS_0)$, the segment immediately preceding it a length of $(TS_1 - TS_0) + \Delta s$, the third but last a length of $(TS_1 - TS_0) + 2\Delta s$, . . . etc., and the first segment again a length of $[(TS_1 - TS_0) + (n - 1) \cdot \Delta s]$. In other words the length of the entire thorax, being the sum of individual segments, should be the sum of an arithmetical series whose first term is $(TS_1 - TS_0)$ and whose last term is $[(TS_1 - TS_0) + (n - 1) \cdot \Delta s]$. This can be put as

$$T_n = n \cdot (TS_1 - TS_0) + \frac{n(n - 1)}{2} \cdot \Delta s \quad (2)$$

where T_n is the total thoracic length at stage n ; and $(TS_1 - TS_0)$, the constant length of the newest segment (or the length of the first segment when in stage c). Taking Δs as 0.0097 mm. and $(TS_1 - TS_0)$ as 0.03 mm., T_n for successive values of n can be calculated. These calculated values are compared with the observed values for thoracic length in Table II; the largest discrepancy is only approximately 5 per cent, and the original suggestion is thus shown to be fact, i.e., every thoracic segment grows in length for a constant amount Δs , in each stage of the thoracic period.

A similar approach can be employed to analyze thoracic changes in width. While in stage 0 thoracic length T_n is also 0, the anterior width of the presumptive thorax is already 0.142 mm. (C). In stage 1, the width of the first segment is 0.144 mm. (Table I), and the initial increase $(W_1 - C)$, analogous to $(TS_1 - TS_0)$ in equations 1 and 2, is therefore 0.002 mm.; the anterior width of the presumptive second segment is again $C = 0.142$ mm. (each segment after stage c being regarded as a short cylinder). In succeeding stages, the width of the first segment increases 0.002, 0.004, 0.005 mm. . . . etc. (Table I). The increments per stage are then not constant, as they were for segmental length, but the figures suggest that the increases of the increments per stage might be constant. If the increment in stage 2 were 0.003 instead of 0.002 mm., the increase Δw over the initial increment $(W_1 - C)$ would be 0.001, and $(W_1 - C) + \Delta w$ would represent the increase in width during stage 2. Similarly $(W_1 - C) + 2\Delta w$ and $(W_1 - C) + 3\Delta w$ would indicate the increases during stages 3 and 4 respectively. In general,

$$(W_n - W_{n-1}) = (W_1 - C) + (n - 1) \cdot \Delta w \quad (3)$$

would be true, where $(W_n - W_{n-1})$ represents the increase in width of the first segment during stage n . The total width increase of the first segment during the first n stages would then be the sum of an arithmetical series whose first term is $(W_1 - C)$ and whose last term is $[(W_1 - C) + (n - 1) \cdot \Delta w]$, for similar reasons as in thoracic length; or

$$W_n - C = n \cdot (W_1 - C) + \frac{n(n-1)}{2} \cdot \Delta w \quad (4)$$

and

$$W_n = C + n \cdot (W_1 - C) + \frac{n(n-1)}{2} \cdot \Delta w \quad (5)$$

TABLE II

Calculated and observed magnitudes of certain larval body regions, in millimeters

Stage No.	Thoracic length		Width of 1st thoracic segment				
	Observed	Calculated	Observed	Calculated			
1	0.030	0.030	0.144	0.144			
2	0.070	0.069	0.146	0.147			
3	0.122	0.119	0.150	0.151			
4	0.185	0.178	0.155	0.156			
5	0.242	0.247	0.162	0.162			
6	0.311	0.325	0.167	0.169			
7	0.390	0.413	0.179	0.177			
8	0.480	0.511	0.191	0.186			
9	0.600	0.619	0.209	0.196			
10	0.733	0.736	0.225	0.207			
11	0.861	0.863	0.245	0.220			
	Length of post-abdomen		Length of a post-abdominal segment		Width of 12th segment		
	Observed	Calculated	Observed	Calculated	Observed	Calculated	
	12	(0.29)	(0.28)	(0.041)	(0.04)	0.150	0.147
	13	(0.325)	(0.30)	(0.054)	(0.05)	0.160	0.154
	14	0.38	0.36	0.06	0.06	0.170	0.163
	15	0.45	0.45	0.08	0.075	0.185	0.174
	16	0.58	0.57	0.09	0.095	0.190	0.187
	17	0.73	0.72	0.12	0.12	0.200	0.202
	18	0.91	0.90	0.15	0.15	0.210	0.219
	19	1.13	1.11	0.18	0.185	0.230	0.238

Taking for $(W_1 - C)$ and Δw the values 0.002 and 0.001 mm. respectively, W_n has been calculated for successive values of n , and the comparison with the observed values is shown in Table II. The percentage discrepancies are greater than those observed for thoracic length, but nevertheless insignificant in view of the greater difficulty of taking accurate measurements of entities of so much smaller magnitude. It is to be concluded that the width of the first thoracic segment grows similarly as the length of the thorax, i.e., by adding, in each stage, another term

of an arithmetical series in which consecutive terms differ by a constant amount Δw .

It must now be shown that other thoracic segments also increase in width according to equations 4 and 5; actual measurements for these segments have not been taken, but the proof can be arrived at indirectly. It is known from observation that the lateral thoracic contours are straight lines converging posteriorly. The angle of taper α (Fig. 2) is always expressed by

$$\tan \alpha = \frac{W_n - C}{2T_n} \quad (6)$$

and this angle, on calculation, is seen to be very nearly constant for successive values of n . For $n = 1$ and $n = 11$, $\tan \alpha$ equals 0.033 and 0.045 respectively; the average from all eleven values is 0.039, corresponding to an angle of $2^\circ 18'$, $\pm 15'$. Since the contours are then straight lines, with a constant taper in all thoracic stages, the taper of individual segments must also be constant and identical, i.e., $(W_n - W_{n-1})/2TS_n$; as the length TS_n of a given segment in a given stage can be shown to be equal to the length, in the preceding stage, of the segment immediately anterior to it, an analogous equality must obtain for the width of a segment, for the taper in each case must be identical. In other words, when the width of the first segment is W_n , the width of the succeeding segment is W_{n-1} , in the same stage; this proves however, by extension, that all thoracic segments must increase in a manner identical to the first, since W_n and W_{n-1} represent sums of the same arithmetical series as that in equation 5, W_n containing one term more than W_{n-1} .

The segmental abdomen during the thoracic phase maintains a constant length ($A = 0.249$ mm.) and a constant anterior width ($C = 0.142$ mm.). The posterior width WU , identical to the "width of the urosome," however increases (Table I). The angle of taper β , therefore, expressed by

$$\tan \beta = \frac{C - WU_n}{2A}, \quad (7)$$

decreases. Stated in other words, the convergence of the abdominal contour-lines gradually diminishes. A stage will eventually be reached at which the thoracic and abdominal contours will form continuous straight lines, the thoracic contours having a constant taper (equation 6); at this time

$$\tan \alpha = \tan \beta$$

and

$$\frac{W_n - C}{2T_n} = \frac{C - WU_n}{2A} \quad (8)$$

from which WU_n can be calculated, all other terms being known. WU_n from equation (8) is 0.123 mm.; the value of WU_n closest to this in Table I is 0.125 mm. in stage 11. It follows therefore that the thoracic and abdominal contours become continuous straight lines as the end of the thoracic period of development is reached.

For analytical purposes thoracic shape during the thoracic period can be regarded as a regular cone from which the tip was cut off (frustrum of a cone). Dorso-ventral extent at any level would be very nearly equal to the lateral width

at that level. The diameters of the end faces of the frustum can thus be assumed to be W_n and W_1 respectively, and since the length of the frustum is always given by T_n , the volume and the surface area of the thorax can be approximated by the use of known geometrical formulae. If the volume V_1 of the first segment is known the total thoracic volume V_n at any stage can also be calculated from a sum-of-a-series equation, of the general form

$$V_n = n \cdot V_1 + \frac{n(n-1)}{2} \cdot \Delta v, \quad (9)$$

which must obtain, since both length and width changes are governed by such equations. Furthermore, Δs and Δw are obviously related mathematically to Δv . In sum, if the initial size and shape of the thorax ($n = 1$), and the values Δs and Δw are known, the size and shape of the thorax at any further thoracic stage can be predicted.

The abdominal phase of development

Abdominal growth.—At the beginning of the abdominal period, the segmental abdomen starts to grow in length, having been constant before. During stages 12 and 13 the abdominal increases are 0.08 mm. per stage, or almost exactly $8 \times \Delta s$ (Table I); since the initial abdominal length at the beginning of stage 12 (or at the end of stage 11) is 0.249 mm. or approximately 8×0.03 mm., it follows that during the genital period each 0.03 mm. portion of the segmental abdomen grows an amount Δs per stage. In other words, the segmental abdomen behaves as though it were already cut up into its eight segments, and each of these hypothetical segments has the same antero-posterior growth potential as thoracic segments when first laid down, viz., increasing Δs per stage after having a length of 0.03 mm. If the 12th segment were laid down in the manner in which thoracic segments are formed, it would reach stage *c* at a length of 0.03 mm. But after stage 11 the entire segmental abdomen has already started to grow, at a rate of Δs per stage per 0.03 mm. Thus at the end of stage 12 when the 12th segment reaches stage *c*, it will be $0.03 + \Delta s$, or 0.04 mm. instead of 0.03 mm. long; the entire segmental abdomen should then be eight times 0.04, or 0.32 mm., and the post-abdomen 0.28 mm. long. Analogously during stage 13, each 0.04 mm. portion of the segmental abdomen will now add an amount Δs , so that segment 13 when in stage *c* will be 0.05 mm. and the entire segmental abdomen eight times 0.05, or 0.40 mm. long. At this point the genital region should be 0.10 (2×0.05) mm. and the post-abdomen 0.30 mm. long. Actual figures in Table I support such an interpretation rather well, and the conclusion is justified that during the genital period the segmental tissue of the abdomen acquires the same growth potential in length as that of equivalent amounts of thoracic tissue during the thoracic period.

The genital region continues to grow in length at the indicated rate, as the data in Table I tend to show. The post-abdomen would similarly do so, were it not for the fact that another change in the mode of growth occurred at the end of stage 13. Successive post-abdominal lengths P_m from stage 13 on are 0.325, 0.38, 0.45, 0.58 mm. etc., in other words the increments are increasing. A sum-of-a-series expression, similar to that for thoracic width changes, fits these figures very

closely, i.e.,

$$P_m - P_0 = (P_1 - P_0) \cdot m + \frac{m(m-1)}{2} \cdot \Delta p \quad (10)$$

and

$$P_m = P_0 + (P_1 - P_0) \cdot m + \frac{m(m-1)}{2} \cdot \Delta p \quad (11)$$

where P_m represents the total post-abdominal length for stages 14 to 19; P_0 , the initial length at the end of stage 13; P_1 , the length at the end of stage 14; m , the successive integers from 1 to 6; and Δp , the increments per stage over the initial increase ($P_1 - P_0$). The theoretical value for P_0 was previously seen to be 0.30 mm., and with 0.06 and 0.03 for ($P_1 - P_0$) and Δp respectively, the calculated values for P_m compare well with the observed ones (Table II).

If equation (11) is divided by six, the growth formula for individual segments is obtained, since each of these segments is one-sixth of the entire post-abdomen;

$$PS_m = PS_0 + (PS_1 - PS_0) \cdot m + \frac{m(m-1)}{2} \cdot \Delta(p) \quad (12)$$

PS_0 , PS_1 , and $\Delta(p)$ are 0.05, 0.06 and 0.005 mm. respectively, and ($PS_1 - PS_0$) is therefore 0.01, or very closely Δs ; thus the initial increase of the presumptive segments 14 to 19, at the beginning of the post-abdominal period, is identical to the increase of these tissues during stages 12 and 13, and this increment is then augmented by a constant amount $\Delta(p)$ in each subsequent stage. What is responsible for this change in the mode of growth of post-abdominal segments? It is more than likely that non-formation of appendages is related to this, inasmuch as newly formed tissue will not be diverted for the establishment and subsequent growth of appendage buds; augmented growth of the segments would therefore be facilitated. It can now be stated in general, that while body segments are formed, length increments per stage for all segments are constant, but the increments may be added to an initial length as in thoracic and genital segments, or to an initial increase of length, as in post-abdominal segments.

As in thoracic segmentation, the anterior width of the segmental abdomen has the constant value $C = 0.142$ mm., during the abdominal period. This value is the anterior abdominal width at the end of stage 11, and the anterior width of the presumptive 13th segment at the end of stage 12. The 12th segment, by this time, has attained a width of 0.15 mm. (Table I), and in succeeding stages this width increases to 0.16, 0.17, 0.185 mm. . . etc. As for thoracic width the increases are found not to be uniformly constant, and a sum-of-a-series expression again approaches the data best, i.e.,

$$WA_r - C = r \cdot (WA_1 - C) + \frac{r(r-1)}{2} \cdot \Delta wa \quad (13)$$

and

$$WA_r = C + r(WA_1 - C) + \frac{r(r-1)}{2} \cdot \Delta wa \quad (14)$$

where WA_r represents the width of the 12th segment at a stage r of the abdominal period; ($WA_1 - C$), the initial increase in width during stage 12; Δwa , the increase in width, per stage, over the increment during the preceding stage; and r ,

the successive integers from 1 to 8. If for $(WA_1 - C)$ and Δwa 0.005 and 0.002 mm. respectively are taken, the calculated values for WA_r compare well with the observed ones (Table II).

Other abdominal segments can be shown to follow a similar mode of growth in width. The lateral contours being straight lines, the angle of taper β is expressed by

$$\tan \beta = \frac{WA_r - WU_r}{2A_r} \quad (15)$$

where A_r is the length of the entire segmental abdomen, i.e., genital plus post-abdominal lengths, and other values as before. $\tan \beta$, when calculated from Table I for successive values of r , centers about the average of 0.036 ± 0.004 ; in other words, the abdominal taper does not only remain constant during the abdominal period, but this taper is also practically identical with that reached by the segmental abdomen at the end of stage 11 (cf. above, equation 8).

Unlike thoracic segments, which start development at stage c with the same length as that of more anterior segments at stage c , the abdominal segments begin development at a length identical with that of more anterior segments at the same time. In maintaining a constant taper, the initial increase of any presumptive thoracic segment over the width C is always expressed by the first term of the series applying to thoracic width (equations 3, 4, and 5), and the later a segment arises the fewer terms of the series can it add to its width during the thoracic period. Since abdominal segments have now also been shown to maintain a constant taper, and since their lengths at stage c are equal to those of more anterior segments already beyond stage c , an analogous relation must similarly exist for segmental width; namely, the initial increase of a presumptive abdominal segment over the width C must be identical to the width increase experienced by other abdominal segments at the same time. If $(WA_1 - C)$ in equation (13) represents the initial increase of segment 12, then $(WA_2 - WA_1)$ would do similarly for segment 13. In other words, the width of both segments follow the same series, but the second term for segment 12 becomes the first term for segment 13; the third term for segment 12, similarly, becomes the first term for segment 14, etc., and the eighth and last term for segment 12 is the first and last term for segment 19. Thus as with thoracic segments, the later an abdominal segment arises the fewer terms are added to its width, but while the width increases of thoracic segments start with the same and end with consecutive terms, those of abdominal segments start with consecutive and end with the same terms of the series.

It should be observed parenthetically that equation (13) may have a slightly different constant Δwa for the genital and post-abdominal segments respectively, reflecting the different modes of growth in length of these two groups of segments; or, if the constant is actually identical the lateral abdominal contour would theoretically not be an exact continuous straight line, but rather two straight lines with slightly different taper, joined between segments 13 and 14. In either case, the difference would be so small as to be unnoticeable in practice; with the present techniques of observation and measurement, a single series relation holds for both groups of segments, and even if two separate series could be established with finer means, the principle of growth in width as outlined above would nevertheless hold.

As for segmental growth in length, a general conclusion can now be stated for

growth in width, viz., width increments per stage for all body segments are constant, and the increments are always added to an initial increase in width. The combined generalization is also true, that total segmental mass increments per stage are constant, and the increments are added either to initial masses or to initial increases of mass.

Thoracic growth.—One of four possible reasons could *a priori* be advanced in an attempt to account for the differential size changes in the thorax, such that the 5th segment ultimately becomes largest, during the abdominal period: i.e., either the segment rudiments in stage *a* differ in initial size but follow the same growth curves; or the analogous converse; or either the rudiments have both equal initial size and identical growth curves; or the analogous negative. Since for all thoracic segments four stage-intervals elapse between stage *a* and stage *c*, length and width magnitudes at stage *c* are identical, and the increments per stage, no matter at which segmental stage, are identical (i.e., Δs), only the conclusion is admissible that thoracic segment rudiments have equal initial sizes and follow growth curves of the same shape. Under such conditions there are two factors which must be held responsible for the observed growth of thoracic segments, i.e., the time lag in the formation of consecutive segments, and segmental age. The time lag fully accounts for the regular gradation of segmental sizes at the end of the thoracic period and for the constant taper of the thorax; as will be demonstrated below, the influence of this original time lag carries over importantly into the abdominal phase, and this, together with the factor of segmental age, can indeed be made the basis for a consistent interpretation of the manner of thoracic growth.

Data in Table I show that thoracic length remains constant during the genital phase. Hereafter the values for length fit the equation

$$T_m = T_{m-1} + (T_1 - T_0)_m - \frac{m(m-1)}{2} \Delta s \quad (16)$$

where T_0 and T_1 represent thoracic length at the end of stage 11 and stage 14 respectively, and m , as before, the integers from 1 to 6. With 0.86 and 0.92 mm. for T_0 and T_1 , and Δs as before, successive calculated values for T_m are 0.92, 1.03, 1.18, 1.36, 1.56, and 1.77 mm., significantly close to the observed data; the increases per stage are therefore 0.06, 0.11, 0.15, 0.18, 0.20, and 0.21 mm., and the differences between the increases are seen to diminish in a regular manner.

The scheme in Table III will account for such a series of increases. The figures in this table represent multiples of Δs and they show the length increase of the indicated segment during the indicated stage. Sums of figures in vertical rows, multiplied by Δs , indicate the increases of the entire thorax during the given stages, and successive sums are seen to be equal to the values for the increases per stage as calculated from equation (16). Horizontal sums, multiplied by Δs , give the total increments of any thoracic segment during the post-abdominal period. This scheme is reproduced somewhat differently in Table IV, in which the figures, multiplied by Δs , indicate directly the size of any of the 19 body segments at any of the 19 developmental stages; vertical sums have meanings analogous to equivalent sums in Table III.

It will be observed that all formulae previously deduced in connection with length increases are inherent in the figures in Table IV; observational data are

also incorporated. For example, the first segment when reaching maturity in stage 7 has a length of 0.09 mm. (cf. Table I). Succeeding thoracic segments must also mature at this size, in consequence to the equality of their growth curves; thus segment 5 is shown to mature in stage 11 when the 19th segment appears in stage *a*, and segment 11 in stage 19, in conformity to the observational data in Table I. The scheme in Table IV also shows well the successive segmental proportions in the thorax during the abdominal phase. In stage 16, segments 1 and 2 are longest, in stage 17 similarly segments 2 and 3, etc.; maximal segmental length thus shifts caudad, fully corroborating observation.

Segmental growth of the thorax as indicated in the table can be interpreted provided two assumptions are postulated, i.e., (*a*) a segment can no longer grow by regularly increasing amounts after having passed through 14 segmental stages,

TABLE III

Scheme of segmental increments, in multiples of Δs , in the thorax during the post-abdominal phase of development

	Stage						Total segmental increases
	14	15	16	17	18	19	
Segment							
1	0	1	1	1	1	1	5
2	0	1	2	2	2	2	9
3	0	1	2	3	3	3	12
4	0	1	2	3	4	4	14
5	0	1	2	3	4	5	15
6	1	1	1	1	1	1	6
7	1	1	1	1	1	1	6
8	1	1	1	1	1	1	6
9	1	1	1	1	1	1	6
10	1	1	1	1	1	1	6
11	1	1	1	1	1	1	6
Total thoracic increases	6	11	15	18	20	21	

counted from stage *c*, and (*b*) a segment, in order to grow by increasing amounts at all, must have matured within the first 6 segmental stages of its existence, counted from stage *c*. These two provisions constitute the limiting conditions of segmental age.

Table IV reveals that only the first 5 segments fulfill the second condition; segments 6 to 11 would also have matured in 6 stages of their individual existence, were it not for the fact that no thoracic growth takes place during the genital period, and maturation of the posterior thoracic segments is therefore delayed by two stages. Thus only the first five segments would be able to grow by increasing amounts, whenever such growth was made possible. It has been shown previously that at the beginning of the post-abdominal phase, the post-abdomen ceases to grow by constant increments and begins growth by increasing increments, with an initial

increase during stage 14 equal to that of stage 13. Apparently the phenomenon of increasing increments at this time is not confined to the post-abdomen but also affects thoracic segments, subject to the limiting provisions stated above. Thus the first five segments have an initial increase equal to the increment during stage 13, viz. 0; segments 6 to 11, not fulfilling condition (b), simply continue at their former constant rates, viz., Δs per stage (cf. data in Table III, under increases during stage 14). From here on, the first five segments augment their increases by Δs in every stage, until their 14th segmental stage is passed; then, by assumption, the increment of the 14th segmental stage can no longer be augmented, but

TABLE IV

*Scheme of growth of body segments, in multiples of Δs
(a refers to segmental stage a of any given segment)*

	Stage																		
	Thoracic phase										Genital phase		Post-abdominal phase						
Seg- ment	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1	3	4	5	6	7	8	9	10	11	12	13			13	14	15	16	17	18
2		3	4	5	6	7	8	9	10	11	12			12	13	15	17	19	21
3			3	4	5	6	7	8	9	10	11			11	12	14	17	20	23
4				3	4	5	6	7	8	9	10			10	11	13	16	20	24
5	a				3	4	5	6	7	8	9			9	10	12	15	19	24
6		a				3	4	5	6	7	8			9	10	11	12	13	14
7			a				3	4	5	6	7			8	9	10	11	12	13
8				a				3	4	5	6			7	8	9	10	11	12
9					a				3	4	5			6	7	8	9	10	11
10						a					3	4		5	6	7	8	9	10
11							a					3		4	5	6	7	8	9
12								(a)				4	5	6	7	8	9	10	11
13									a				5	6	7	8	9	10	11
14										(a)				6	7.5	9.5	12	15	18.5
15											a				7.5	9.5	12	15	18.5
16												(a)				9.5	12	15	18.5
17													a				12	15	18.5
18														(a)				15	18.5
19															a				18.5

is retained as a constant increment till growth stops altogether. Thus segment one has increased its increment of zero by Δs at the end of stage 15 (Tables III and IV); but at this point its 14th segmental stage has already been passed and hereafter only a constant increment of Δs per stage is possible. Segment 2 on the other hand is younger than segment one, being laid down in stage c with a time lag of one developmental stage. By the end of stage 15, therefore, when segment one has just passed its 14th segmental stage, segment 2 has only passed its 13th segmental stage and its increment of Δs during stage 15 can be augmented once more by Δs ; when the 2nd segment has passed its 14th segmental stage, its in-

creases in subsequent stages will therefore be $2 \Delta s$ per stage. Similarly, segments 3, 4, and 5, each being one stage younger than the preceding segment, are able to augment their increments by Δs 3, 4, and 5 times respectively, before they complete the 14th segmental stage. Segment 5 in consequence is as long as segment 4 at the end of stage 19, but the former will continue to grow at a rate of $5 \Delta s$ per stage, while the rate of the latter can only be $4 \Delta s$ per stage; at any time after stage 19 therefore the fifth segment will be longest.

Analogous changes occur with regard to thoracic growth in width, and the thoracic cone-frustum of stage 11 gradually assumes the shape of a barrel, with the "waist" at segment 5 after stage 19. The dorsal thoracic curvature of the animal, arising similarly after stage 11, can also be interpreted as a result of differential segmental increases in a dorsal direction, according to a scheme resembling that in Table III.

The genital segments have been noted to mature, i.e., to form a broodpouch, in stage 18. Table IV reveals that at the end of this stage, segment 12 has just completed its 6th, and segment 13, its 5th stage of segmental development, counted from stage *c*. Thus both segments fulfill one of the two age conditions assumed for thoracic segments; the fulfillment of the other might be expected. Observation proves that this is actually so. Genital segments of older larvae are known to bulge considerably beyond the general abdominal contour, giving them a knobby appearance. This could not be possible if the constant increases observed up to stage 18 were maintained any further; rather, after stage 18 the initial increment of an increasing rate will again be equal to the increase during the stage just passed, viz., Δs , and during a 20th stage this increment will be augmented by a given amount, during a 21st stage by twice this amount, etc., till the 14th segmental stage is passed.

The post-abdominal segments have previously been shown to grow by regularly augmented increases as soon as they are laid down. But since these segments bear no appendages, stage *c* for them is equivalent to attainment of maturity, as already observed above. Maturity thus proves to be an important temporal threshold for all body segments, and the statement that augmented growth will occur in any mature segment, provided maturity was reached in a definite time, has general application; the concept of segmental maturity is apparently not only a working hypothesis, as has been assumed at the start, but seems to have real biological meaning.

There is no doubt that the scheme of growth here presented describes correctly the actual events of later thoracic development; but the assumptions, while justified by the interpretations they allow, still remain to be explained. Only tissue culture studies will be able to reveal why segments not matured in the first 6 stages of existence are at too early a stage of development, and why segments after 14 stages of existence are at too advanced a stage to do more than keep up a constant rate.

Growth of appendages; integration of segmental development

In the preceding section it has been reasoned that segment rudiments in the thorax have equal initial size and identical growth curves; observation tends to confirm not only this but also that equal-sized rudiments develop for all body segments. It can be assumed that in these rudiments certain tissue masses (ap-

pendage rudiments), initially also of equal size and of equal growth capacity in equal times, differentiate independently towards the establishment of appendage buds. Such buds however never appear in the post-abdomen, and when they appear in other regions they may develop into swimming appendages or into a broodpouch. In the evidence presented in Tables I and IV, an important clue can be found to at least one of the factors preventing serial analogy despite the observed serial homology in appendageal development.

Every thoracic appendage rudiment reaches the bud stage after an interval of four developmental stages. The segment as a whole is at stage *c* at this point, and the appendage buds of any thoracic segment must be of identical size, due to the identity of initial size and of growth capacity for all appendage rudiments, and of identical shape, since every thoracic segment at stage *c* has identical proportions. Enough appendageal tissue has apparently been manufactured, during the four preceding stages, to initiate the development of a swimming appendage.

When a genital segment reaches stage *c*, 4 + and 5 developmental stages have elapsed since stage *a*. The appendage rudiments therefore have time to manufacture proportionately more appendageal tissue, at the same intensity as that of thoracic rudiments. If the genital segments in stage *c* had larger sizes, proportionate to the longer time interval available, the appendage buds of genital segments would have the same size and shape as those of thoracic segments. However, both the length and the width of genital segments are greater in stage *c* than the size which would be proportionate to the longer time of formation. The length of any thoracic segment when laid down is 0.03 mm., for example, and four developmental stages have elapsed since stage *a*; the length/time ratio is thus 0.03/4. In genital segments this ratio is larger, viz., 0.04/4 + and 0.05/5, and analogously for width. Appendageal tissue in genital segments can therefore not be developed in sufficient quantity, in proportion to segmental size, to produce appendage buds of dimensions equal to those of thoracic buds, even though more time is available. Genital buds will thus be relatively smaller and flatter, and the amount of appendageal tissue manufactured will be spread more thinly over the presumptive appendage region; the quantity of tissue present per unit area is apparently already below the threshold necessary for the formation of comparatively specialized swimming appendages, and only enough tissue is available to initiate the formation of a relatively simple sac.

In post-abdominal segments at stage *c* the size/time ratio becomes progressively larger still, and appendageal tissue consequently cannot even accumulate in quantities sufficient to form a bud.

After the appendage buds are laid down, an appendage retains a definite size-proportionality to the segment bearing it. When, for example, the thoracic contour is a straight line, during the thoracic phase of development, the line joining the tips of the appendages on one side is also a straight line, and, as with the segments themselves, the time lag in bud formation accounts for the taper. Similarly, as the thorax gradually becomes barrel-shaped in the abdominal phase, the transformation is reflected in differential length increases in the appendages, and when the appendageal tips on one side of the body are joined by a line, the result is an analogously barrel-shaped contour.

From the above analyses, the following integrated sequence of events becomes apparent with regard to segmental development.

Shortly before hatching segmental rudiments of equal size begin to be formed, at a rate of one per developmental stage; with a time lag of four stages, segments are constricted off in posterior succession, all with constant initial sizes and increasing by a constant amount during each stage. As the first segment reaches maturity, the rate of segment rudiment formation increases to two per stage. Rudiments are laid down at this rate till the newest rudiment appears at the posterior end of the segmental abdomen which latter had so far maintained a constant length. The last formed rudiment happens to be the 19th and by this time, 11 segments have been constricted off, five of which have already matured.

The process of rudiment deposition and segment constriction could be assumed to go on at length, were it not for the fact that the "end" of the animal has been reached. This is apparently the cue for a general change in the mode of growth. The entire segmental abdomen begins growth, increasing as yet equal amounts per stage, and the thorax ceases to grow. After two genital segments of equal size are formed another general change occurs, to the effect that hereafter any segment maturing within a definite time may grow by augmented increases, as described in detail above. This type of growth is maintained, in each segment in which it takes place until the 14th segmental stage is passed, whereupon the total increment of the 14th stage is reproduced without further increase in each succeeding stage. Segments not matured within the required time continue to grow by constant increments. The eventual result of this varied manner of growth, maintained up to sexual maturity, is the barrel-shape of the thorax, the presence of a dorsal thoracic curvature, the knobby appearance of the broodpouch segments, etc.

DISCUSSION

Throughout the present analysis of metamerism in *Artemia salina*, the time scale employed was that of developmental stages, defined as the number of body segments present. It must be eminently realized that this is a scale of relative, biological time. Events in nature take place in a space-time continuum, and to *Artemia* equivalent happenings in space, i.e., the establishment of segments, must be correlated to the passage of equivalent units of (relative) time, i.e., what here had been called "stages." In hours and minutes, segment formation occurs of course not in equivalent times, since the phenomenon is dependent on the environment on the one hand, and on changes in growth rates with age on the other. *Artemia* and other similarly primitive forms are particularly suited for a ready identification of relative time, but in segmented animals of greater complexity, as well as in non-segmented groups, "equivalent happenings in space" cannot be picked out with comparative ease, and it will be more difficult to tell what the relative time scale actually is; but that it is intrinsically present in biological phenomena has already been acknowledged by others. Thus Needham (1942), after briefly reviewing the pertinent literature, states:

"Mouse time must bear the same, or a similar, relation to elephant time as mouse spatial magnitudes to elephant spatial magnitudes. Indeed, unless the time factor is brought into account, we may understand morphological similarity, but we can never hope to understand physiological, still less embryological, similarity."

Measurements on *Artemia* in absolute time would never have brought to light the truly amazing simplicity of the laws of segment formation, as given by the series and the sum-of-series formulae, and in terms of relative time these formulae assume a simple biological meaning, viz., (a) that equivalent spatial events take place during equivalent relative times, and also (b) that equivalent spatial events take place in tissues of equivalent relative age. For illustration, the thorax during the thoracic period of development may be considered, where the increments per stage of $(TS_1 - TS_0)$ and $(W_1 - C)$ (equations 1 and 3) are indeed equivalent and constant, and where every other segment grows similarly in this same manner; summation of the increments must then result in the sum-of-series expression. Analogous interpretations, based on the idea of spatial and temporal equivalence can be adduced in every other case in which the formulae hold, i.e., virtually for the entire period of segmental development. Before and after this period, relative time is of course still operative, but its expression is latent, inasmuch as its passage is not paralleled by morphological events clearly identified as equivalent. The same would be true for the majority of living organisms, but it can be asserted with a fair amount of logical conviction, that if and when it will be possible to make explicit the relative time scales of living organisms as a whole, size increments in relative time units will be found to be equivalent, and series formulae of linear, quadratic, and perhaps even of higher degree will be found to hold.

It should in general be useful to have a specific term to distinguish relative biological time from absolute duration; the concept as a whole might be called "biochronism," and the relative time scale could be said to have one "biochron" as its unit. Also, in order to transcend the usual connotations of "growth rate," "biochronal rate" could be substituted. Whenever in the text above "increase per developmental stage" was mentioned, "increase per biochron" was really implied. In this connection, the type of analysis in the present report is clearly different from "allometric," "heterauxetic," or "heterogonic" inquiries. The term "morphometry" is suggested to indicate generally any quantitative appreciation of organic size, shape, and time as an integrated dynamic pattern. While it is realized that apologies are in order, more or less categorically, for the introduction of any new term into present-day biology, it should be kept in mind that new terms become unavoidable as different methods of inquiry and fresh fields of study appear.

Two immediate issues have not been touched on at all in the present analysis. First, what determines the changes in the mode of segmental growth at the end of both the thoracic and the genital periods? That the changes occur is fairly definitely established, and this would support the view that division of segmental development into periods is real, i.e., physiological as well as morphological. But beyond that, speculation into the nature and history of the changes would be futile, for lack of direct evidence. Secondly, and this is the fundamental question in the study of metamerism, why are segments formed at all? It will readily be admitted that even an attempt to answer this problem can only be made after a great deal more is known about segmentation phenomena as a whole.

Excepting these two questions however, the final size and shape of *Artemia* nevertheless has here been accounted for in terms of initial body proportions much as Berrill (1941) has done for the ascidian, *Botryllus*. When copepods, crayfish, and other diverse crustacean forms of higher evolutionary rank are considered, similarly in possession of a barrel-shaped thorax and a straight tapering abdomen,

it is perhaps justifiable to reflect that Crustacea as a group might have evolved with a single and basic geometrical pattern of growth.

SUMMARY

1. Growth and the dynamic pattern of segment formation in excysted larvae of *Artemia salina* have been quantitatively studied. The final shape of *Artemia* at sexual maturity can be accounted for in terms of initial shape at hatching.

2. In analyzing the pattern of metamerism, the stages of development are gauged by the number of body segments present. Growth during the entire period of segment formation is found to be governed by arithmetical series and sum-of-series relations, implying that growth increments per stage over either initial sizes or initial increases are constant and identical for thoracic, genital, and abdominal segments, respectively. Later transformations of larval shape, resulting in the barrel-shape of the thorax, the presence of a dorsal thoracic curvature, the knobby appearance of the genital segments, and the presence of a straight tapering abdomen, are accounted for analytically on the basis of concepts concerning the age of segments and the time lag involved in segment formation.

3. The presence, absence, and the difference of structure of appendages are shown to be determined, at least in part, by the size of segments when first laid down, and by the time available for appendage rudiments to form appendageal tissues.

4. The time scale employed in the analysis of the segmentation pattern in *Artemia* is interpreted to be a relative, biological one, and the meaning of the series formulae with regard to this relative scale is illustrated. The notion of "biochronism" is introduced, as a general concept applying to biological events in relative time.

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ELECTRON MICROSCOPE OBSERVATIONS OF THE TRICHOCYSTS AND CILIA OF PARAMECIUM

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In previous publications, electron micrographs have been shown of trichocysts (Jakus, 1945) and of cilia (Schmitt, Hall, and Jakus, 1943). Recently we have re-examined both these organelles using the shadow-casting technique of Williams and Wyckoff (1945). The new technique shows structural detail with improved clarity and reveals some features not previously visible in specimens prepared in the conventional manner.

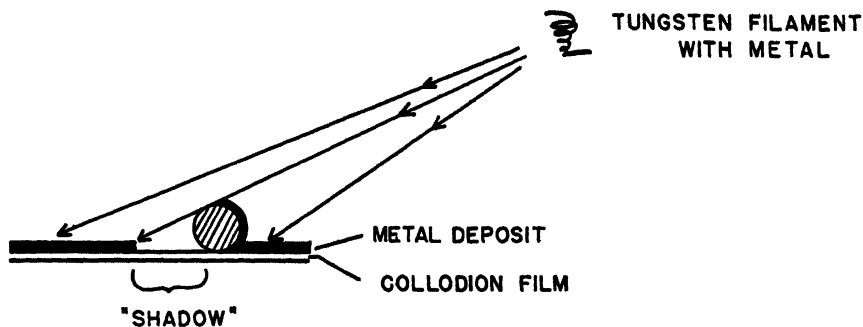


FIGURE 1. Diagram of shadow-casting technique.

The shadow-casting technique is illustrated diagrammatically in Figure 1. A specimen is placed in a vacuum bell-jar containing a conical tungsten filament in which are placed some small pieces of a suitable metal such as chromium. When the filament is raised to a high temperature by the passage of an electric current the metal evaporates, travelling in straight lines and depositing on the specimen as indicated. Structures projecting above the surface of the supporting film cast permanent shadows to the "leeward" and intercept metal to the "windward." Specimens are then examined in the electron microscope in the usual manner. In positive prints the shadows appear bright because they represent relatively transparent regions in the object. It is customary, therefore, to prepare micrographs as negative prints so that the shadows will appear darker than the background.

TRICHOCYSTS

The structure and properties of the trichocysts of *Paramecium* have been described in a previous paper (Jakus, 1945). In electron micrographs, the discharged trichocyst consists of a sharply-pointed tip and an elongated, cross-striated shaft with a periodicity of about 550 Å. The cross-striated structure appears to be a

thin membrane formed by the lateral aggregation of fine fibrils. The tip, in contrast to the shaft, is quite opaque. The reason for this opacity was not obvious.

Further information about the morphology of the dried extruded trichocyst is obtained from electron micrographs of shadowed specimens (Fig. 2). The tip is seen to be a compact structure which stands up from the film and is not flattened to any great extent as a result of dehydration. The contour of its shadow indicates that it is shaped somewhat like a golf tee. In contrast to the tip, the dried shaft is very flat, as is evident from the short shadow it casts. The cross striations previously observed are enhanced by the metal, indicating that the surface has a regularly corrugated contour. The elevated regions correspond to the darker bands in both untreated trichocysts and those stained with phosphotungstic acid. Other details of structure observed previously may also be found in some shadowed trichocysts. These are the fine longitudinal striations of the shaft membrane and the larger periodicity (2,200 Å) frequently noted along the shaft. The latter may appear simply as a slight further intensification of every fourth dark band, suggesting that these ridges have a somewhat higher elevation than do the others.

In some specimens the pointed tip appears regularly cross-striated, if the amount of metal deposited has not been excessive and the orientation of the tip is approximately parallel to the direction of deposition. This banding has not been seen in either stained or unstained specimens and, while it is readily visible in the original micrographs of shadowed tips, it is not considered to be of sufficient clarity for reproduction. Although relatively constant in any one tip, the spacing varied from 280 to 365 Å in the different tips measured and had an average value of about 300 Å. This is to be compared with the average period of about 550 Å in the trichocyst shaft.

CILIA

The cilia of *Paramecium* are shed quite readily if the cell is injured and both intact cilia and fragments are observed frequently in preparations of trichocysts. Each cilium consists of a bundle of fibrils (about eleven in number), extending the full length of the cilium (Fig. 3). The diameter of the dried fibrils lies between 300 and 500 Å. It may be of significance that both the number of fibrils and their diameter are in close agreement with the corresponding values observed in the sperm tails of numerous animal forms (Schmitt, Hall, and Jakus, 1943).

In fixed preparations (for example, with OsO_4), the component fibrils usually adhere to form a compact bundle, while in unfixed cilia they separate to a greater or lesser extent. They are clearly defined in shadowed specimens. Usually the separation of fibrils is not complete and they remain in close contact near the end of the cilium which was attached to the cell. Here they appear sometimes to be joined into two closely adjacent bundles.

It is not evident what holds the fibrils together in the living cilium. No spiral sheath similar to that observed in mammalian sperm tails (Schmitt, Hall, and Jakus, 1943) or in *Euglena* flagella (Brown, 1945) has been seen. If a sheath does exist, it must be very fragile and easily ruptured. In some cilia, a rather poorly-defined cross-striation has been noted, particularly in two or more adjacent fibrils. This striation appears to be unlike that of clearly cross-striated proteins and, if it is not an inherent periodicity in the fibril, it may represent the remnants of some binding or enveloping structure.



2



3

FIGURE 2. Trichocysts from *Paramecium*, shadow-cast with chromium. $\times 16,000$.
FIGURE 3. Cilium from *Paramecium*, shadow-cast with chromium. $\times 11,000$.

SUMMARY

Electron micrographs of shadow-cast trichocysts of *Paramecium* show that the dried trichocyst shaft is flattened on the supporting film, while the pointed tip is apparently more resistant to collapse on dehydration. Accentuation, by the metal, of the cross striation previously observed in the shaft indicates that the periodicity is accompanied by corrugation of the dried surface. A cross striation in the tip is also visible in some micrographs of shadow-cast specimens. In the few cases where the periodicity could be measured, the average spacing was about 300 Å, as compared to about 550 Å for the well-defined shaft striation.

In electron micrographs of shadow-cast specimens of *Paramecium* cilia, the component fibrils are seen with greatly increased clarity.

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HYDROSTATIC PRESSURE EFFECTS UPON THE SPINDLE FIGURE AND CHROMOSOME MOVEMENT. II. EXPERIMENTS ON THE MEIOTIC DIVISIONS OF TRADESCANTIA POLLEN MOTHER CELLS

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INTRODUCTION

Hydrostatic pressure increments are known to reduce progressively the rigidity of plasmagels and the viscosity of plasmasols. Eventually complete solation results. Marsland (1939 and 1942) has been able to formulate what appears to be a general quantitative law on the basis of a considerable volume of work with very diverse material. He has found that with each increment of 1,000 lbs./in.² hydrostatic pressure, the relative rigidity or viscosity decreased to 76 per cent of the initial value. This applied no matter whether the cytoplasm of amoebae, *Arbacia* eggs, or *Elodea* was being studied. Furthermore, these direct effects have always proved very rapidly reversible when the pressure was released. The subsequent pattern of cell events, however, has sometimes been found to have been changed by new reorganization patterns (cf., Pease, 1940, 1941).

In the first paper of this series (Pease, 1941), experiments were reported in which advantage was taken of these known effects of hydrostatic pressure to study the first cleavage division spindle apparatus in *Urechis* eggs. The material was not well suited for this sort of work, and some interpretations were open to question. However, the following facts were clear and significant. 1) Pressure could so affect the cell that no trace of the spindle figure appeared in the fixed preparations, and presumably the spindle had been completely liquified. 2) The pressures destroying the spindle blocked all anaphase movement. 3) The chromosomes aggregated in clumps (originally thought to be vesicles) under lower pressures than were required to block anaphase movement. 4) Numerous cytasters appeared in material given a brief recovery period before fixation. 5) Peculiar "half-spindles" developed *de novo* within cytasters whenever the latter came in contact with nuclear material. 6) By their very nature, the half-spindles lacked "continuous fibers" since only one pole was involved, and also there were no "interzonal fibers." 7) Yet there was ample evidence that such half-spindles were functional in moving chromosomes, and even recently-formed nuclei with membranes were at least deformed, and probably moved, by them. The role of cytoplasmic components in the spindle was stressed (perhaps unduly), and the role of the "traction fibers" minimized (perhaps incorrectly as will be seen later).

To find out whether or not nuclear gels behaved in the same manner as cytoplasmic gels when hydrostatic pressures were applied, the extraordinary equational meiotic division in *Steatococcus* spermatocytes has been studied in unpublished work by the author. In these cells the spindle is formed inside the nuclear membrane,

and the anaphase movement nearly completed, before the nuclear membrane disintegrates. In this case, there can be no question but that the whole spindle apparatus is of nuclear derivation. It was found that sufficiently high pressures destroyed it by liquefaction, and anaphase movement was blocked. The spindle re-formed once more when the pressure was removed and the cells allowed a short recovery period. Thus the physiological action of hydrostatic pressure appears to be qualitatively identical in gels of nuclear and cytoplasmic origin.

For the present work, *Tradescantia* pollen mother cells (PMC) were selected as material for several reasons. The spindle is characterized by relatively enormous "traction fibers" going to the poles from comparatively large and easily visible kinetochores. The cells have the advantage of a small number of chromosomes which are relatively large. The only important disadvantages are the impossibility of getting controls which necessarily divide at the same time as the experimental material, and the extreme difficulties (which proved insuperable with pressure techniques) of actually observing the divisions *in vivo* (cf., Shimakura, 1934).

The material was collected and prepared at Stanford University, and the author is indebted to Dr. Reed Rollins of that institution's botany department for technical advice on handling procedures and for the plants which were used. The material was studied mostly at Columbia University before the war interrupted the work. Dr. F. Schrader, Dr. S. Hughes-Schrader, and Dr. H. Ris followed its course with interest, enthusiasm, and valuable suggestions. Dr. C. W. Metz of the University of Pennsylvania also contributed excellent comments on an early draft of the manuscript.

MATERIAL AND METHODS

The half dozen *Tradescantia paludosa* plants used in these experiments possessed six pairs of chromosomes. They were derived from a common stock. The anthers were prepared by separating the connective which joins the two lobes. One lobe was then fixed as a control just at the time of pressure application to the other lobe. The bisection of the anthers with a small lance could be accomplished easily without rupturing the anther lobe walls. The lobes were handled and finally mounted in a 7.4 gm./100 ml. saccharose (Merck, C. P.) solution which Shimakura (1934) has found to be isotonic with *Tradescantia* pollen mother cells.

The pressure bomb used in these experiments held a half dram homeopathic vial, and was so designed that it could be opened very rapidly. After filling with sugar solution and a few anther lobes, the vial was sealed with "Parafilm" wax sheet held in place with a rubber band. The experimental material was always kept under the desired hydrostatic pressure for a one hour period. In a few experiments the material was fixed 30 minutes after the release of pressure which allowed time for some recovery. But in most of the experiments, the pressure was released, the bomb opened, and the fixative added within one minute. Preliminary experiments had shown that there was no appreciable reorganization within that short time limit.

Experiments were performed using 1,000 lb. pressure increments from 1,000 to 6,000 lbs./in.², and with 8,000, 10,000, and 15,000 lbs./in.² Control experiments were performed giving identical treatment, but at atmospheric pressure, and at the relatively low pressure of 150 lbs./in.²

Bouin's fixative, to which 3 per cent urea was added, was used throughout. For study, eight micra sections were prepared, and stained by Heidenhain's hema-

toxylin method. Both mordanting and staining were prolonged (never less than 5 hours each), and the sections were destained in saturated picric acid in such a fashion that considerable stain remained in the cytoplasm. There was a good deal of shrinkage, but the cytoplasmic differentiation (particularly of the spindle) was good.

RESULTS

Effects upon the first division spindle

The first division spindle was particularly sensitive to a critical hydrostatic pressure that was found to be between 4,000 and 5,000 lbs./in.² Even after 4,000 lbs. had been applied, the spindle figures looked essentially normal. There was no reduction in the length or diameter of "traction fibers" (compare Fig. 28 with Figs. 25 and 26). However, many of the "continuous fibers" had apparently been lost for the net effect was a more diffuse looking spindle mass with fewer and less conspicuous continuous fibers. The abnormalities of chromosome movement under even the lower pressures prevented any adequate study of "interzonal connections," but occasional examples that looked normal have been found after 4,000 lbs./in.²

In striking contrast were the results after 5,000 lbs. had been applied. The traction fibers were then reduced in length and in diameter so that they appeared as delicate structures (Fig. 30). Small numbers of faint and very thin continuous fibers were usually visible, although not always. Ordinarily 6,000 lbs. pressure obliterated the spindle completely, but in a small fraction of the cells a fine residual fiber structure remained visible. Figure 31 is a photograph of the heaviest and most extensive fibers which have been observed in material fixed after an exposure to this pressure. It must be emphasized that this is an entirely atypical cell. No sign of continuous fibers has been seen after exposures to 8,000 lbs., and it was the very rare cell which showed indications of traction fibers. When visible, as in Figure 33 (arrows), they were thin and short. No oriented fiber structure of any sort was ever observed after exposures to 10,000 or 15,000 lbs./in.²

In summary, it can be said that the first division spindle looked essentially normal after treatments with 4,000 lbs./in.² pressure, but was profoundly affected by 5,000 lbs. This demarkation was really very sharp!

Effects upon the second division spindle

The spindle of the second meiotic division was considerably more resistant to hydrostatic pressure than that of the first division. The spindles appeared nearly normal after 4,000 lbs./in.² pressure, and after 6,000 lbs. the spindles of some cells did not seem to be greatly affected. After 6,000 lbs. pressure there was a considerable individual variability in different cells, even within the same anther lobe. The best spindles were somewhat fainter than normal, and the fibers seemed generally thinner, but they sometimes extended from one pole to the other. After 8,000 lbs. pressure there were occasionally evidences of traction and continuous fibers, although they were always thin and faint if present. No fiber structure was ever visible after pressures of 10,000 lbs. or more.

It thus appears that the second division spindle withstood nearly 2,000 lbs./in.² more pressure than the spindle of the first division. It will appear later that the pressure required to block anaphase movement was similarly proportional.

It may also be noted here that there was a little evidence that the spindles of the somatic cells in the connective were even more resistant to pressure, and were not entirely destroyed unless pressures in excess of 8,000 lbs. were applied.

Effects upon the chromosomes—fusion

Increasing hydrostatic pressures made the chromosomes progressively more "sticky" and "soft." Chromosomes tended to aggregate in fused masses. In Figure 27 a metaphase plate is shown, fixed just after the release of 2,000 lbs. pressure. It will be noted that there are stained "bridges" connecting all of the chromosomes. At this low pressure, the bridges were, on the average, only slightly heavier than comparable bridges which could be found in controls of the proper stage. However, they persisted much longer than normally, well into the anaphase stages.

When pressures of 3,000 lbs. or more were applied, the inter-chromosomal bridges tended to become much thicker, and entirely out of the range of normal variation. Figure 32 shows such connections in a cell fixed just after the release of 6,000 lbs. pressure. With progressively higher pressures, there was an increasing tendency for the fusion of chromosomes into a single mass. This can be seen in Figures 33 and 34. The extreme condition was reached at 15,000 lbs./in.² when it was nearly always quite impossible to recognize individual chromosomes. This is well shown in Figure 36.

It must be emphasized that the preceding description and the photographs are typical of cells to which the pressure was applied in late metaphase stages. When the pressure was applied to early metaphases, the chromosomes showed a much greater degree of fusion for corresponding pressures. Of considerable importance must have been the proximity of chromosomes, and probably also the initial presence of thin connections. The existence of some movement in the low pressure range may have aided the process.

Not only were metaphase chromosomes fused together by treatment with hydrostatic pressures, but a comparable effect was observed with late diakinesis chromosomes before the nuclear membrane broke down. Here the chromosomes are apparently normally kept separate from one another by gel structure within the nucleus, for nucleoplasm strands showed clearly enough in fixed preparations. These strands continued to be visible until pressures of 6,000 or 8,000 lbs./in.² were applied. As long as they were present the chromosomes kept apart and did not fuse. After the higher pressures the strands were no longer visible, and the chromosomes were all in a single clump together. But, as with the metaphase chromosomes, the individual chromosomes did not lose their visible identity until pressures of 15,000 lbs. were applied.

At metaphase, the chromosomes were not only found fused laterally in the plane of the equatorial plate, but the homologous chromosomes were also fused together so that their separation was greatly complicated. This was very obvious when first division anaphases fixed just after the release of 3,000 or 4,000 lbs. pressure were studied. Practically every cell showed evidences of fusion with bridges that were often long and massive (cf., Figs. 1-12). Such bridges always stained just as the chromosome proper with hematoxylin (Fig. 39), and the larger ones, at least, were stained by the Feulgen reaction. These bridges were frequently between homologous chromosomes, but also commonly involved lateral fusion with non-homologous chromosomes.

Even more massive bridges were found in the second meiotic division material subjected to the higher pressures which still allowed a good spindle to exist. Then, after 6,000 lbs./in.² pressure, most or all of the chromosomes were frequently so fused together that they nearly lost their visible identity. However, the mass of chromosomes often would be strung out from one end of the cell to the other (Fig. 15).

It should be noted that the chromosomes of somatic cells showed the same type of fusion. These have occasionally been seen in the tissue of the connective, and Figure 41 shows one bridge out of a total of three present in such a cell fixed just after the release of 4,000 lbs. pressure.

Effects upon the chromosomes—rounding

It should be emphasized that all of the fusion bridges between chromosomes had rounded outlines. This shows well in Figures 27 and 32, and suggests a considerable plasticity.

In addition, the chromosomes as a whole tended to round up under the higher pressures. This was most obvious in the second division chromatids which were V-shaped with relatively long and thin arms. After 3,000 lbs. pressure there was very little noticeable change in shape even though there might be some fusion (Fig. 13). However, after 4,000 lbs. there was a striking alteration. The chromatids were then decidedly thickened and shortened (Fig. 14). This tendency became more pronounced with increased pressures (Fig. 15, 6,000 lbs.).

The short and thick chromosomes of the first meiotic division were not as suited for study, but the same tendency was obviously present. Particularly after 10,000 lbs., when the identity of individual chromosomes could still be seen, they were decidedly shortened and rounded except at the kinetochore region (Fig. 34).

Effects upon the chromosomes—the spindle attachment region

The first meiotic division material gave the impression that 1,000–3,000 lbs./in.² pressure allowed a greater extension of the attachment region of the chromosomes than was normal (compare Fig. 26 with 25). More particularly, this region of some chromosomes was extended far beyond what could be found in the controls. The attachment region gave the impression of being unduly short in the material exposed to 4,000 lbs. pressure. An attempt to measure statistical samples was decided upon.

In Table I the mean extensions of the attachment regions of first division chromosomes are given for pressures up to 4,000 lbs./in.² There were, of course, real difficulties in measuring such small distances, but errors should have cancelled out in the averages. While no great reliance should be placed on the absolute values, they certainly indicate the general trend.

The measurements were made with a filar micrometer. In each group, 50 measurements were made at random, excepting that only cells in anaphase were selected, and individual chromosomes that had not yet separated and left the metaphase plate were measured. The micrometer hair was moved up to a chromosome until it just touched the distal tip of the kinetochore (indicated by the arrows in Figs. 25 and 26), and a reading made. Then the hair was swung across the field, and moved back in the other direction until the hair just touched the base of the attachment

stalk which was ordinarily rather well defined from the body of the chromosome by its relative translucency. Then a second reading was made. The difference measured the length of the stalk plus the width of the hair in the micrometer. The hair width was measured in the same way in relation to a fixed point, and this value was subtracted from all of the measurements. The figures were then converted to micra. The control measurements actually used for comparison were combined from data upon the control anther lobes of the 1,000 and 3,000 lb. experimental material, and a control anther which was left mounted in the bomb for one hour before fixation, but without pressure.

It is to be concluded that the mean length of the attachment stalk was definitely increased by pressures from 1,000 to 3,000 lbs./in.², and it has also been found that there is no overlap in the extreme extensions between control cells and experimental cells exposed to this pressure range. With 4,000 lbs. pressure the mean extension was significantly less than in the controls, and the greatest extensions found after this treatment did not even approach the maxima found in the controls.

TABLE I

Pounds pressure	Mean extension in micra	Percentage increase in length	Percentage overlap with control mean
control	0.85		
1,000	1.4	59.4	2
2,000	1.2	38.6	6
3,000	1.2	42.9	4
4,000	0.68	-19.9	22

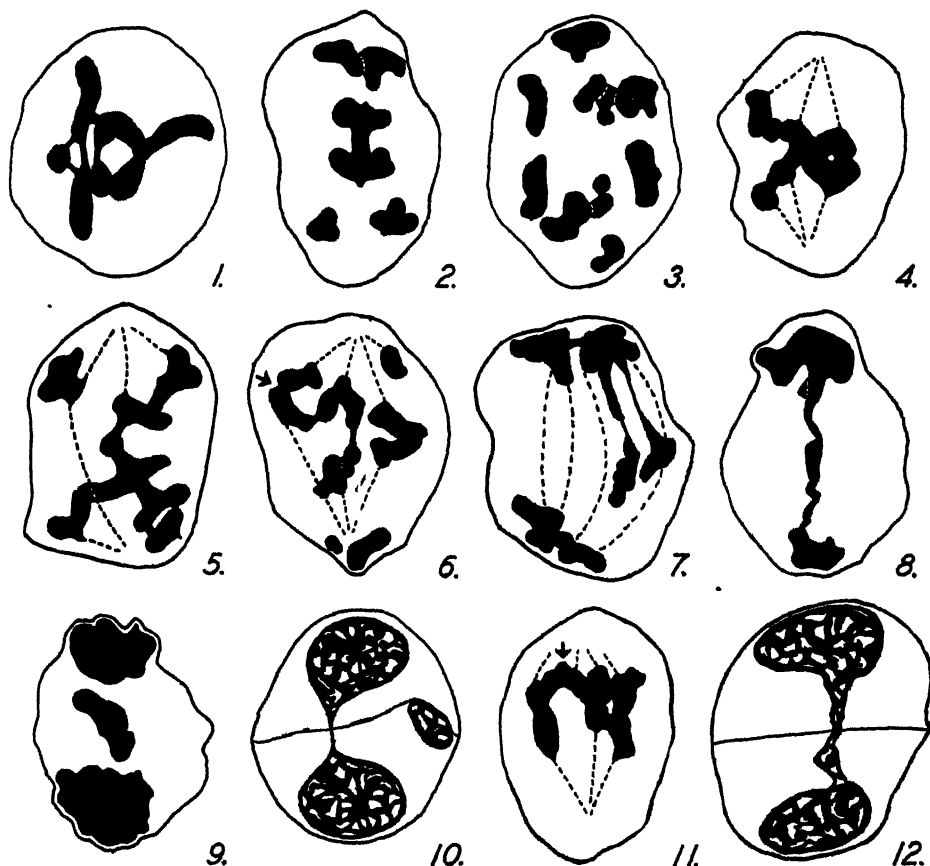
The distance between the tip of the kinetochore and the base of its stalk is given in the second column. In the experimental series, 50 measurements were made at random, excepting only that early anaphase cells were selected. The control average, however, is a combined average of three sets of measurements upon different material. The mean percentage increases in length are based upon figures carried to the third decimal place. The last column gives the percentage of measurements which overlapped the mean of the control.

Effects upon the chromosomes—chromonemata

We have already seen that late prophase and metaphase chromosomes fused together and rounded up under the influence of hydrostatic pressure. This, however, only applied to condensed chromosomes. Uncondensed early prophase chromosomes did not seem to be affected by even the highest pressures employed. This agrees with the findings of Pease and Regnery (1941) who were unable to detect any effect of 15,000 lbs./in.² pressure upon *Drosophila* salivary chromosomes which are similarly uncondensed. It must be admitted that no detailed study has been made of the early prophase chromosomes. While there was certainly no general clumping, it is possible that very local fusions could have been overlooked, but there was no indication of shortening or thickening.

An "accidental experiment" gave further information, and additional reason for believing that the chromonemata were not affected by hydrostatic pressure. An anther lobe which had been pricked was exposed to 15,000 lbs./in.² pressure for one hour and was then rapidly fixed in the usual fashion. The surrounding sugar solu-

tion had entered the anther, and apparently was somewhat hypertonic. All of the cells were slightly plasmolized and had more or less swollen chromosomes. In one small section of the anther, conditions were such that the spiral structure was visible. Figures 37a and b are photographs of one of these early anaphase cells, and it is obvious that the spiral structure was unaffected. Oddly enough there was no tendency for the chromosomes to fuse under these circumstances.



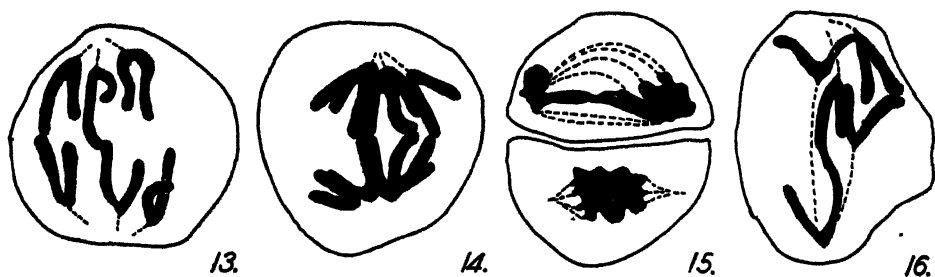
First division cells. Figures 1-10 are of sections from material which was fixed just after the release of 4,000 lbs./in.² pressure. Figures 11 and 12 are of sections fixed just after the release of 3,000 lbs. pressure. The broken lines represent traction fibers except in Figure 7 where they represent the pathways of "continuous fibers." All of the chromosomes visible were not necessarily included.

Abnormalities of chromosome movement under pressure

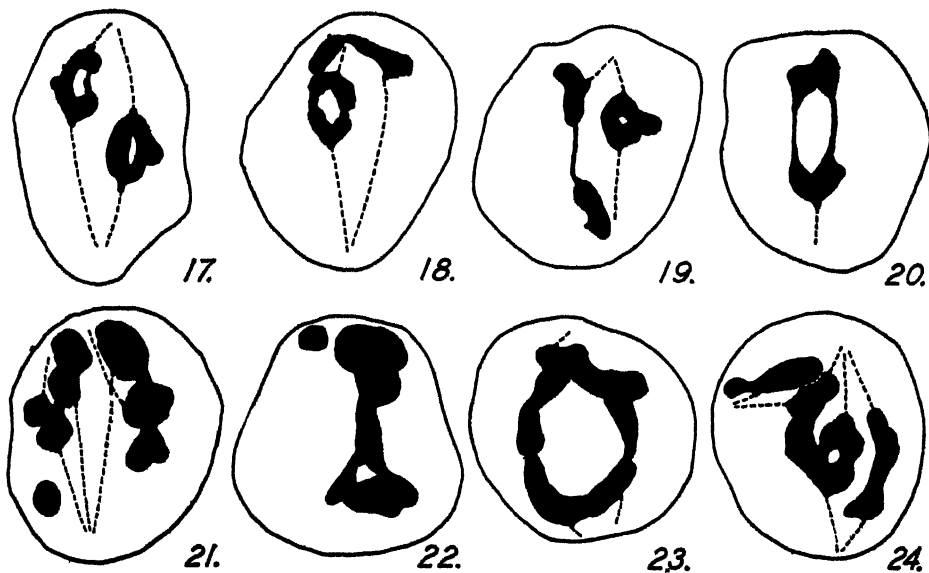
Because of the fusion of metaphase chromosomes, even by relatively low pressures, their ultimate distribution to the two spindle poles was usually very abnormal whenever anaphase movement took place during the pressure treatment. The particular pattern which resulted apparently depended upon the balance between ana-

phase forces and the local resistances of whatever fused bridges happened to be present. Greater or lesser fusions might occur between homologous chromosomes and, laterally, between non-homologous chromosomes. Almost any conceivable variation in the resulting pattern could be found in all degrees. Some of the more interesting variations which have been seen are included in Figures 1-15, which are also perfectly typical of material exposed to 3,000 or 4,000 lbs. pressure.

Homologous chromosomes might be so extensively fused that separation could not occur. Such pairs of chromosomes, fused as in Figure 2 in the metaphase plate



Second division cells. Figure 13 is from material fixed just after the release of 3,000 lbs./in.² pressure; Figure 14, after 4,000 lbs. pressure; and Figure 15, after 6,000 lbs. pressure. Figure 16 is from recovery material, fixed 30 minutes after the release of 10,000 lbs. pressure. The broken lines indicate traction fibers except in the upper cell of Figure 15 in which they indicate the pathways of the "continuous fibers." Not all visible chromatids were necessarily included.



Figures 17-24 are all from first division recovery material which was fixed 30 minutes after the release of 10,000 lbs./in.² pressure. The broken lines indicate traction fibers. Not all visible chromosomes were included except in the last three figures.

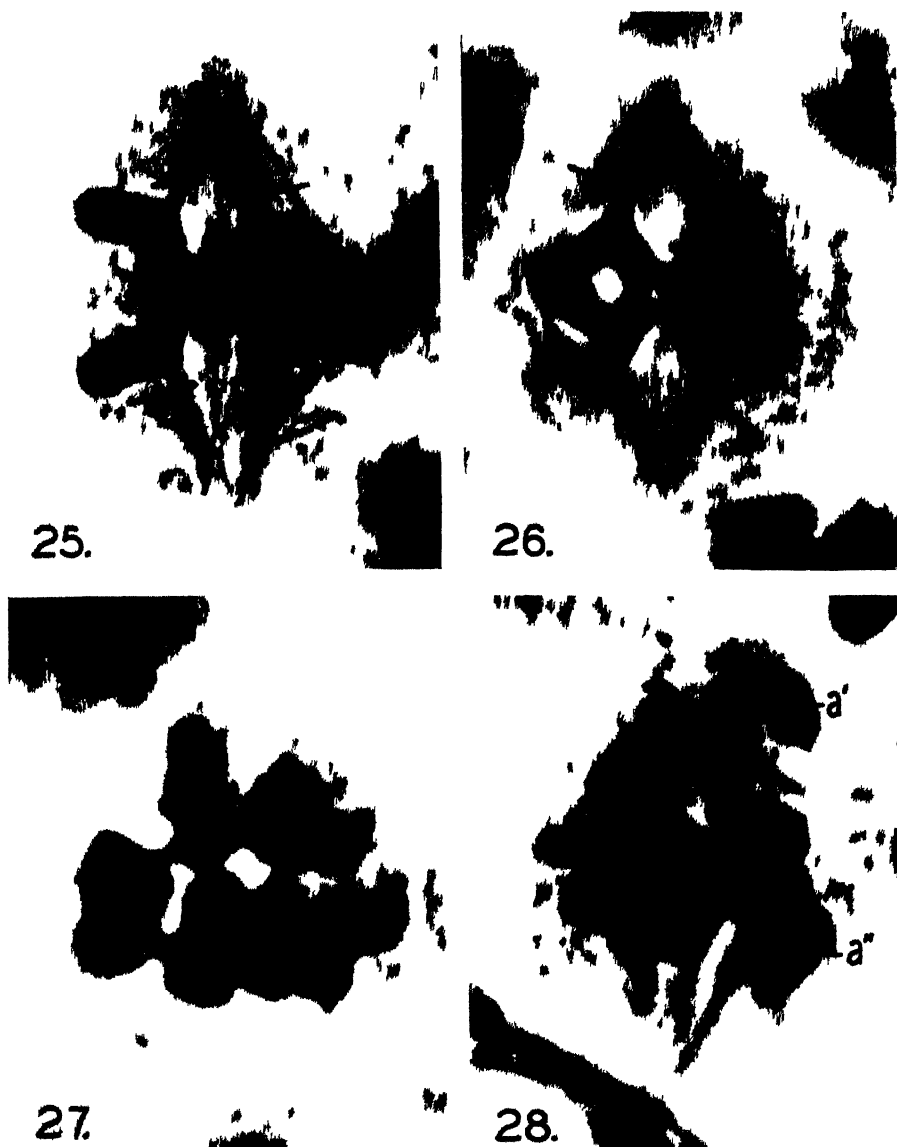


Figure 25 is a first division early anaphase control exposed in the bomb for an hour (but without pressure) before fixing. Figure 26 is of a cell fixed just after the release of 2,000 lbs./in. pressure. Figure 27 is a metaphase plate of the same material. Figure 28 is of a cell fixed just after the release of 4,000 lbs. pressure and note the anaphase separation of the homologous chromosomes a' and a'' . The small arrows indicate the distal ends of the kinetochores. The magnification of these and the following photographs is approximately $\times 3,000$.

region, would presumably have remained there, and eventually formed micronuclei (Figs. 9 and 10).

Even though there was no lateral fusion with other chromosomes, there might be slight differences in the forces directed towards the two poles, or possibly in the strength of the traction fibers going to opposite poles. An extensively fused pair of chromosomes might then go as a unit to one pole (Fig. 5). Then there would always be an abnormally long, but otherwise normal looking traction fiber (with full thickness) going most of the way across the cell to the other pole.

Figures 4 and 5 show very extensive lateral fusion between non-homologous chromosomes. Such anaphase cells would probably have given rise to extensive bridges in telophase, and between daughter nuclei, such as are shown in Figures 8, 10, and 12.

In Figure 6 the lower member of a pair of homologous chromosomes, indicated by an arrow, was laterally fused with a non-homologous chromosome going to the upper pole. Seemingly it was being carried to that pole in spite of its traction fiber to the other pole.

We have already spoken of the massive bridges which characterized the second meiotic division material exposed to 6,000 lbs. pressure, and which often involved all of the chromatids (Fig. 15). There was less fusion with lower pressures, and the abnormalities more nearly resembled what has just been described for the first division.

The critical pressure blocking anaphase movement

The best evidence for chromosome movement under pressure is certainly the presence of extensive bridging. The author sees no rational way of accounting for the bridges other than to suppose that anaphase movement occurred after the chromosomes established fusions in the metaphase plate and then pulled out the bridging connections.

With this as a criterion of movement, it is possible to state that anaphase movement continued at 4,000 lbs./in.² hydrostatic pressure in the first meiotic division, but was blocked by 5,000 lbs. pressure. No extended bridge has been seen in any cell of this division exposed to 5,000 or more pounds pressure. Nor were there ever signs of asynchrony, or of directionally atypical movements.

It must also be emphasized that abnormal division resulting from fusion characterized practically *every* anaphase cell exposed to 4,000 lbs. pressure. It was also extremely common after 3,000 lb. treatments. Similar abnormalities appeared on a lesser scale after 1,000 or 2,000 lbs., but then the separation was more frequently fairly normal, and characterized only by loss of division synchrony.

In the second meiotic division very abnormal anaphase movement involving massive fusions took place in some cells exposed to 6,000 lbs./in.² pressure (Fig. 15), but none was possible at 8,000 lbs.

Bridging has been found even after 8,000 lbs. pressure in the somatic cells of the connective. Figure 12 is from a somatic cell forming daughter nuclei at this pressure, and two out of a total of five bridges are visible in the plane of the photograph.

In the meiotic divisions, at least, the presence of a good visible spindle was correlated with anaphase movement. When the spindle was obviously considerably af-



29.



30.



32.

Figure 29 is a late anaphase cell from the same material as Figure 28 (exposed to 4,000 lbs/in² pressure) Figure 30 is a cell fixed just after the release of 5,000 lbs pressure Figures 31 and 32 are from material fixed just after the release of 6,000 lbs pressure

fects there were no longer evidences of anaphase movement. This was also probably true of the somatic cells, but they have not been carefully studied. It is clear that movement is most sensitive to hydrostatic pressure during the first meiotic division, withstands nearly 2,000 lbs. more pressure in the second division, and seemingly about 2,000 lbs. more in the somatic cells. This, in turn, appears due to different characteristics of the spindle gels, rather than being due to differential pressure effects upon the chromosomes. For in the first and second meiotic divisions, and probably also in the somatic divisions, the chromosomes seemed affected equally by equal pressures.

Spindle recovery after pressure release

At the time of making these experiments the importance of the recovery stages was largely unsuspected, and relatively little material was gathered. But after one hour exposures to 10,000 and 15,000 lbs./in.² pressures, some experimental material was removed from the bomb and given a 30 minute recovery period before fixing. Many of these cells showed excellent spindles with massive traction fibers (Fig. 38).

Of particular interest is the fact that the traction fibers of these recovery spindles were *de novo* formations. Conclusive evidence of this was afforded by paired homologous chromosomes (still fused as a result of the pressure treatment) which formed traction fibers from both kinetochores that went to the same pole. Figure 39 is a photograph of such a condition. Figure 40a is a drawing of another example. Figure 40b seems further complicated for apparently one traction fiber had to curve around a blocking chromosome before its direction to the "wrong" pole could become definitive. In Figure 40c each traction fiber can probably be considered as having gone to the "wrong" pole so that the original polarity of each chromosome was entirely reversed.

Figures such as those described in the last paragraph were not rare, although out of the ordinary. They were never seen in the controls, nor is the author aware of similar accounts in the literature.

Most commonly the spindle appeared to re-form nearly along its original axis if it is assumed that the metaphase plate was not displaced, and remained as an index of that polarity. The pattern thus usually seemed very nearly normal. However, the long axis of the new spindle was sometimes very oblique to the plate, and presumably to the original spindle axis. In extreme cases a 90° shift was indicated.

Also, not infrequently multipolar spindles were found which were very rare in the control material. Three-pole spindles such as Figure 24 were fairly common, and a few four-pole spindles have been seen. All possible variants were seen with equal or very unequal poles, spaced equidistant from one another, or barely separated.

These several lines of evidence all imply that the spindle was re-formed *de novo*, and was not rebuilt upon residual structure which had survived the pressure treatment and persisted to give a framework. New patterns appeared, and whatever molecules were involved, they were at least rearranged.

The development of the recovery spindle

One can select a series of cells which apparently show the different steps of spindle re-formation after the release of pressure. In some cells fiber structure con-

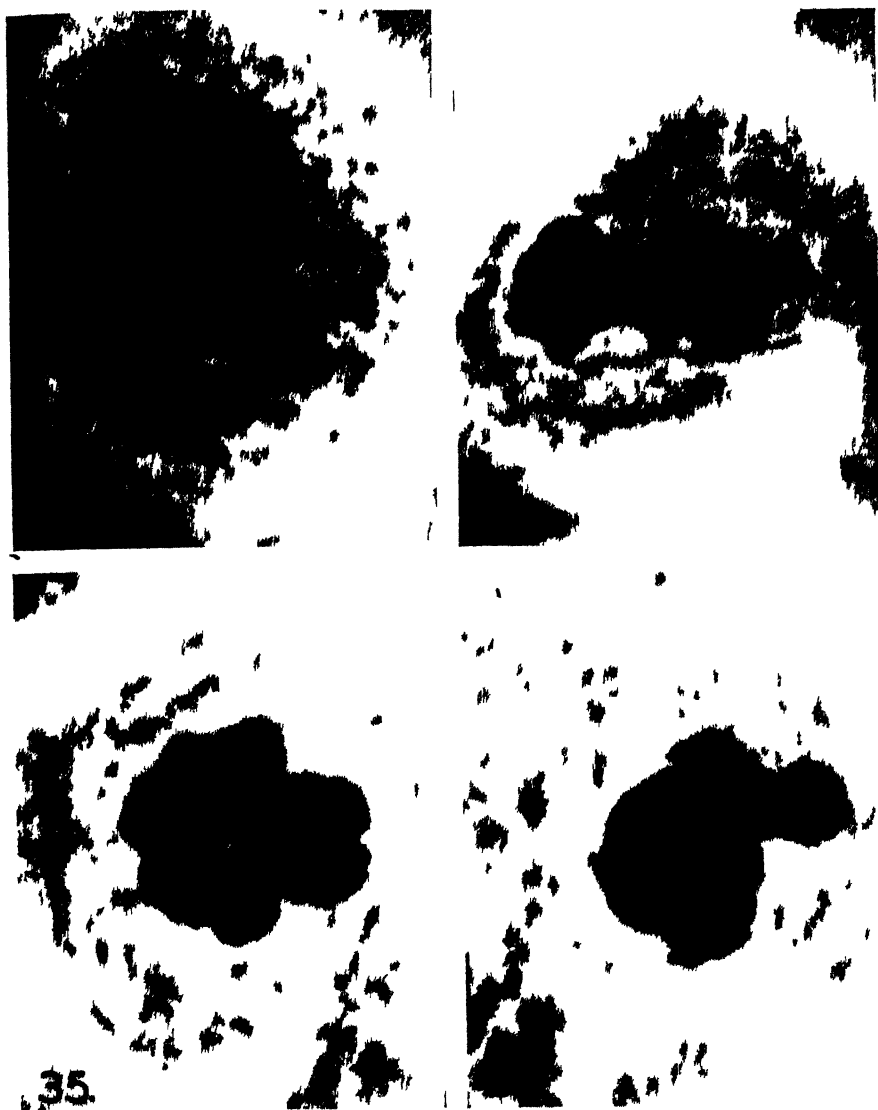


Figure 33 is of a cell fixed just after the release of 8,000 lbs/in² pressure (the arrows indicate very faintly visible traction fibers), and Figure 34 after 10,000 lbs pressure. Figure 35 is of a cell fixed just after the release of 15,000 lbs pressure, and the orientation is thought to be in the plane of the original spindle axis. Figure 36 is from the same material, but sectioned in the plane of the metaphase plate.

sisted of thin fibrils tangled around the clumped chromosomes of the equatorial plate, and without any polar orientation. The fiber direction was roughly circumferential to the enclosed mass of chromosomes (as in a cocoon, Fig. 44). This could be regarded as the first recovery stage.

Many cells showed polarized fibers as in Figure 45a. The section of Figure 45 is oblique to the spindle axis. The focus of Figure 45a is tangent to the slant height of the cone which makes up one-half of the entire spindle (the "surface" of the spindle, so to say). The visible fibers are the continuous fibers of the new spindle. Figure 45b is a lower focus of the same cell. It should be observed that there are no continuous fibers in the center of the cone. Instead, there are only slight indications of traction fibers. The continuous fibers were thus largely peripheral, but the extensive lateral fusion of the chromosomes to make a practically solid metaphase plate probably had much to do with this morphological pattern which was typical of recovery material.

Traction fibers were not seen in cells without polarized continuous fibers. But when the latter had formed, traction fibers could usually be found. In some cells they would be thin and short, in others longer and more massive. Thus the traction fibers appeared to "grow" outward directly away from the kinetochore region, and full thickness was not achieved until they practically reached the poles.

It was possible to find many minor irregularities in the developmental pattern of traction fibers. These resulted whenever the kinetochore pointed in some other direction than directly towards a pole. A graded series could be found, the extreme examples being when kinetochores pointed more or less to "wrong" poles. Invariably the base of the traction fiber extended directly away from the kinetochore, and it did not bend towards a pole until it became associated with continuous fibers. The bend would then be towards the pole less than 90° away from the initial growth direction even if this happened to be the "wrong" pole. It thus looked as though the growth direction was unimpeded until the traction fiber became associated with continuous fibers, and then the further extension of the traction fiber followed the path of least resistance in the pattern expressed by the continuous fibers. Thus the traction fiber even developed around obstructions as in Figure 40b.

The fusion of traction fibers

A very rare situation casts further light on the formation of traction fibers if the interpretation is correct. It was possible to find non-homologous chromosomes in the recovery material which appeared to be bridged across the kinetochore regions. A photograph of such a bridge is shown in Figure 43. These bridges differed from all the other ordinary bridges which have been seen in that they were achromatic. Although they were short, they had exactly the appearance in the fixed and stained preparations that traction fibers had. They certainly gave the impression that they represented fused traction fibers, traction fibers which started to develop from each separate kinetochore in opposite directions, and which grew terminally into each other to fuse end to end.

The author hesitates to emphasize these structures. The material has been thoroughly searched and only two good examples have been seen, plus another which was more questionable because overlying material partially obscured it. There may be good reason for their rarity, for it is obviously an exceptional situation to have two kinetochores pointed directly towards each other. If we accept their

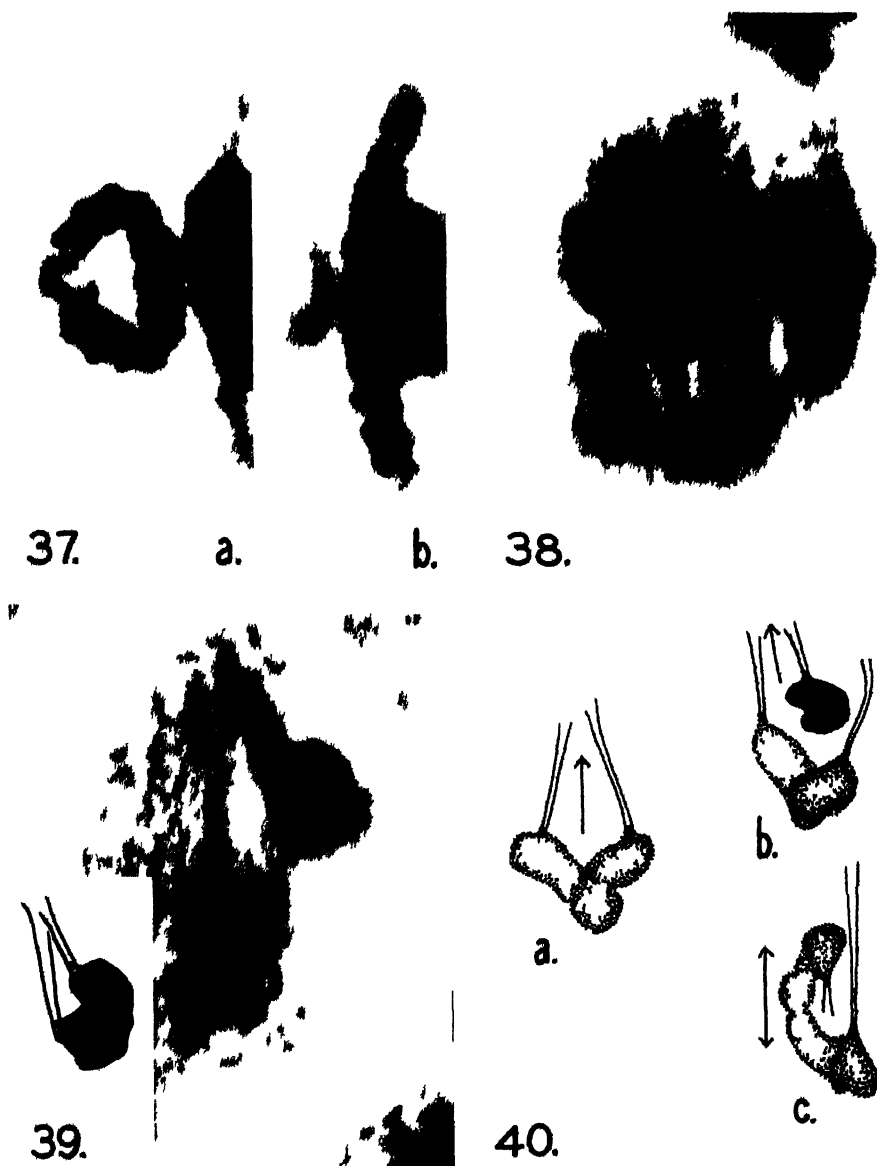


Figure 37 is from a slightly plasmolized cell fixed just after the release of 15 000 lbs/in² pressure (*a* and *b* are different focal levels). Figures 38–40 are from recovery material fixed 30 minutes after the release of 10 000 lbs pressure. In Figure 38 note the bridge, *br*. In Figures 39 and 40 *de novo* recovery traction fibers of fused homologous chromosomes go to the "wrong" pole. The direction of a pole is indicated by arrows in Figure 40.



Figure 41 is of a somatic anaphase cell fixed just after the release of 4,000 lbs./in.² pressure. Figure 42 is of a somatic cell forming daughter nuclei, fixed just after the release of 8,000 lbs. pressure. Figure 43 is from recovery material fixed 30 minutes after the release of 10,000 lbs pressure, and shows achromatic bridging between non-homologous chromosomes (fused traction fibers?). Figure 44 shows an early stage of spindle recovery in material fixed 30 minutes after the release of 10,000 lbs pressure. Figure 45 is from the same material, but spindle recovery is more advanced (*a* and *b* are different focal levels of the same cell).

reality and the above interpretation, however, the implications are of considerable interest, for it means that developing fibers can mutually terminalize each other. Yet there is no effect as far as lateral growth is concerned, and the fibers thicken as normally. There is simply no growing end left. We can say that fibers extend by terminal additions rather than from the kinetochore, or by elongation from within their length.

Having gone this far, we can make another deduction as to the role of the kinetochore in traction fiber formation. We can regard it as an "organizing center" which initiates linear extension and controls fiber diameter. The linear growth is self-perpetuating once started until the fiber reaches a pole, or is terminalized as above. The fiber thickens by further organization at the kinetochore, and additional linear growth parallel to the initially thin fiber, thus adding enclosing layers. The final fiber has a thickness equal to the diameter of the organizing center. The author reiterates that this hypothesis has a slender experimental basis, and depends upon a correct interpretation of three figures.

Chromosome movement in recovery material

There were obvious indications of chromosome movement in recovery material after the spindles re-formed. The movement was abnormal because of strong and persistent fusion bridges, and in many ways resembled the anaphase movement which occurred under low pressures (3,000 and 4,000 lbs.).

Frequently fused pairs of homologous chromosomes were found going to, or after they had reached, a single pole as in Figures 38 and 17. In such cases one traction fiber extended all the way across the cell to the other pole but seemed to be of normal thickness. This type of movement often seemed to be aided by lateral fusion with non-homologous chromosomes as in Figures 18 and 21. Less frequently the fusion between homologous chromosomes was relatively slight, and there would be a partial separation with the formation of more or less long and thin bridges (Figs. 19, 20, and 38, *br.*). Quite frequently very massive bridges were formed involving most if not all of the chromosomes which would be fused together (Figs. 22 and 23). There were no important differences between first and second meiotic division cells (note Fig. 16).

None of the material was allowed a sufficient recovery period so that daughter nuclei formed in cells which began their anaphase movement after the application of pressure. It can be presumed, however, that many of the cells would form only a single nucleus because of an inability on the part of the chromosomes to separate. Other cells would be expected to form bridged nuclei, and probably multiple micronuclei.¹

Chromosome structure in recovery material

The persistence of chromosome fusion in the recovery material would seem to suggest just one possibility—that the initial fusion under high pressure must have been due to at least a partial liquefaction of some chromosomal element, and that the fusion bridges then gelled when the pressure was released. In the recovery material the chromosomes were thus stuck together by very viscous bridges. After examining a great deal of material, the author is of the opinion that it is very doubt-

¹ Pease (1941) definitely found this to be the case in *Urechis* eggs.

ful that fused chromosomes were ever able to separate completely before the formation of daughter nuclei. Most commonly there were few signs of any separation, but even in extreme cases, thin and very long bridges persisted as in Figures 19 and 20. The moderately thick bridges, at least, stained with Feulgen.

There is another, and much more puzzling, aspect of chromosome structure which is brought to light by a study of the recovery material. Even after the release of 15,000 lbs./in.² pressure (which resulted in the very complete fusion of the chromosomes as in Figure 36) the chromosomes regained their visible identity and their approximately normal shape. This tendency can be seen (in 10,000 lbs. material) by comparing Figure 38 with Figure 34, but it is best seen by comparing the long chromatids of the second meiotic division (compare Fig. 16 with Figs. 14 and 15). In regaining the normal shape, the fusion areas must necessarily have been reduced in cross-section, and it is likely that some fusion bridges were lost entirely during this change. The effects of this change were best demonstrated by the separation of the second division chromatids in material recovering from 10,000 lbs. pressure. Extensive separation sometimes occurred, thus differing in degree from the first division. Figure 16 gives an indication of typical difficulties which were essentially the same as in the first division.

Absolute pressure and recovery rate

In *Urechis* egg material Pease (1941) found that the rate of recovery was roughly proportional to the absolute pressure which had been applied. In the *Tradescantia* PMC material we can only compare the effects of 10,000 and 15,000 lbs./in.² pressures. Comparison is subjective, but there was not the slightest doubt but that the cells subjected to 10,000 lbs. pressure showed a much greater amount of recovery of the spindle elements in 30 minutes than the cells exposed to 15,000 lbs. showed in the same length of time. Fully developed new spindles were only rarely found in the 15,000 lb. material, but were common in the 10,000 lb. material. In both, however, the chromosomes had regained their visible identity and approximately normal shapes.

CONCLUSIONS

A single hypothesis readily accounts for most of the manifold effects of hydrostatic pressure upon spindle, chromosomes, and anaphase movement. This supposes that increasing hydrostatic pressures progressively reduce gel rigidity, with liquefaction as the end result. Conversely, after the release of pressure, conditions return to a state such that gel structures can be re-formed once more. There is, of course, an excellent experimental background for this thesis, particularly in so far as it applies to cytoplasmic systems. This has been indicated in the introduction, and has been outlined at greater length in the first paper of this series (Pease, 1941).

It is, however, unfortunate that this work depends upon an interpretation of fixed material. However, we have every reason for believing that the presence of good fiber structures in such material is a good index of oriented gel structure in life. It is only on that assumption that a comprehensive pattern appears, consistent throughout its details. It is true that whenever we have contributory evidence of liquefaction (such as a block of anaphase movement), we do not find fiber structures in the cytological material. Apparently extensive fiber structures are only

precipitated by fixation agents when molecules are at least organized into an oriented pattern and probably also concentrated in a gel.

Spindle structure and formation

In view of the above considerations, it is not surprising to find that the spindle no longer appears in cytological preparations after a critical pressure has been applied before fixation. This is to be interpreted as indicating a liquefaction of pre-existing gel structures, with a consequent loss of molecular organization.

It has been demonstrated that the pattern of the recovery spindle can be very different from that of the original spindle. High hydrostatic pressure seems able to break down the oriented structure of the original spindle so completely that it re-forms *de novo*, and sometimes with a new polarity. In the re-formation of the spindle much the same protoplasmic material may well be used, but the unit molecules or micells are rearranged in a different manner, just as a pile of second-hand bricks might be rearranged to build a new house. This conclusion can probably be accepted as a generalization for it agrees with the findings in *Urechis* eggs which, in their formation of "half spindles," were even more striking (Pease, 1941), with certain other observations on cytoplasmic systems (cf., Pease, 1940), and with general theory.

It is not clear just what does orient the new spindle axis in *Tradescantia* PMC. Cytasters accomplished this end in *Urechis* eggs, and obviously played the important role. These were never observed in the PMC material. Instead, we find a strong tendency for the new axis to coincide more or less with the original. The recovery spindle encountered one unusual difficulty in its organization in that the chromosomes were no longer completely separate entities. After the higher pressures there was usually a continuous plate of fused chromosomes in the equatorial region. Continuous fibers did not, indeed could not, penetrate this obstruction. However, note that homologues were not even found as half spindle components. Continuous fibers were only found sweeping around the blocking mass leaving the core of the spindle devoid of visible oriented structure except for traction fibers. Apparently, therefore, the continuous fibers are entirely a product of the cytoplasm, and are not directly related to the chromosomes. The latter, in fact, are obstacles to be by-passed. This does not, however, preclude the possibility of a generalized interaction between chromosomes and cytoplasm in that the former may "activate" the latter to form gel structures. Such an "activation" was quite definitely shown by *Urechis* eggs recovering from the effects of hydrostatic pressure (Pease, 1941). A more accurate interpretation might be not to stress the continuous fibers as such, but to consider them simply as an index of a more fundamental structural organization of molecules. They thus may signify nothing more than the basic pattern of an extensive gel framework.

On the other hand, the kinetochore apparently quite specifically "organizes" the protoplasm to form the attached traction fiber. This process is partially separable from the development of continuous fibers. We have good reason for believing that developing traction fibers simply follow the path of least resistance in the structural pattern of the bulk of the spindle, which, in turn, is expressed by the distribution of the continuous fibers. Thus the structural pattern of the body of the spindle limits the course taken by the traction fibers as they develop outwards away from the kinetochores. It seems likely that this is a progressive wave of molecular or-

ganization. This view is quite similar to that of Schrader (1932), although based upon different evidence. However, it is fundamentally distinct from that of Belar (1929) who supposed a very different relationship between traction and continuous fiber. Further tentative conclusions on the growth of traction fibers have already been given in describing the experimental results.

The extension of the attachment region in chromosomes subjected to relatively low pressures indicates a real pull by or through the traction fibers. It is almost impossible to imagine that it could be due to "repulsive forces" between the kinetochores for, if that was so, the extension should continue to increase with progressively higher pressures which further soften the chromosomes. Instead, we find the extension to be subnormal while we still have evidence of traction fibers and anaphase movement (at 4,000 lbs. in the first meiotic division). Our conclusion, then, is that the traction fiber is a reasonably stiff gel. No doubt it progressively loses rigidity with increasing pressure, but it has a margin of strength, and there is no important weakness until a pressure threshold is passed. The extension of the attachment stalk is therefore thought due to a pressure effect upon the chromosome itself so that it is softened, and can be unduly pulled out. The subnormal extension at 4,000 lbs. indicates a significant weakness of either the traction fiber or available force. It is interesting for comparison that the centrifuging experiments of Shimamura (1940) with comparable material (*Lilium* PMC) also lead to the conclusion that the traction fiber is a fairly stiff gelled structure. The latter's work seems to the author to be quite conclusive.

Chromosome structure

It seems obvious that some portion of the condensed chromosome tends to be softened, and finally liquefied, by hydrostatic pressures. Since there was no apparent effect upon uncondensed chromosomes, or upon the spirally coiled chromonemata, the portion affected would seem to be the "matrix" (no morphologically separable "sheath" is visible, and presumably more than a sheath would be involved when the attachment region is extended).²

A critical analysis of the data, however, discloses some relationships that cannot yet be interpreted with any assurance of certainty. The normal presence of an attachment stalk, and its further extension under relatively low pressures, suggests that the rigidity of the matrix is normally low, but is further reduced by pressure. One might suppose it to be viscous rather than a stiff gel. While the spindle gels are liquefied by moderate pressures, the matrix is not entirely liquefied until pressures of about 15,000 lbs./in.² are applied when the chromosomes so fuse that they lose their visible identity. Thus a structural viscosity appears to persist and withstand very considerable pressures.

It is a fair assumption that the spindle gels obey Marsland's (1939) law, so that their rigidity is reduced 24 per cent by each pressure increment of 1,000 lbs./in.² Liquefaction then occurs at a critical pressure, when gel linkages tend to break more

² In the first paper of this series (Pease, 1941) chromosome aggregation was described in *Urechis* eggs subjected to hydrostatic pressure. The cytological appearance suggested that a "sheath" was involved in this fusion rather than the matrix. The *Urechis* chromosomes were so small, though, that the details were not visible. In view of the present work it seems more likely that the matrix as a whole was involved.

rapidly than they can be formed. Whereas we can probably apply Marsland's law to the spindle gels, it does not seem applicable to the chromosome matrix, unless we assume that the matrix material has a much lower pressure/rigidity constant than cytoplasmic or spindle gels, i.e., much less than 24 per cent per 1,000 lbs./in.² That other different gels *in vitro* do, in fact, have different constants has been demonstrated by Marsland and Brown (1942).

There is yet another aspect of chromosome structure to be considered. Why is it that with increasing pressures we find chromosomes rounding up and tending to fuse into a single mass? This looks like an interfacial phenomenon to be explained on the basis of surface tension laws. We do not observe this with uncondensed chromosomes. The author does not see how these and related observations can be explained except by the assumption that a true interface does exist between condensed chromosome and surrounding protoplasm (cf., Hirschler, 1942). Many workers do not believe that there is an osmotically active membrane separating chromosome from protoplasm, although this could explain many of the observations of chromosome swelling. However, a real interfacial boundary would not necessarily imply an osmotically active system.

In any case, it can be presumed safely that any intracellular interface would exert only a very low tension, certainly not more than a fraction of a dyne, or the very few dynes, that have invariably been recorded for water/cell interfaces, or intracellular oil/protoplasm interfaces (cf., Harvey and Shapiro, 1934 and Harvey and Schoepfle, 1939). The presence and properties of dissolved proteins would always prevent high values. Thus any interfacial tension at the surface of a chromosome would be so low that complete rounding of the aspherical shape would occur only when both chromosome and surrounding protoplasm were essentially fluid, and practically without structural viscosity. It is only at a pressure of about 15,000 lbs./in.² that the observed effect indicates these conditions as being nearly fulfilled.

The spindle in chromosome movement

It has already been pointed out that there is a direct and definite correlation between anaphase movement and the presence of a good visible spindle. Hence, our outstanding conclusion is that the presence of gel structure in a spindle is essential for anaphase movement. When the gel rigidity is sufficiently reduced, the movement necessarily ceases. Other types of experimentation have less directly led to the same conclusion (cf., particularly the work of von Möllendorff, 1938 and 1939, on the specific effects of chemical agents). On the other hand, hypotheses involving attractive or repulsive forces are well nigh incompatible with the results. It is hard to imagine hydrostatic pressure affecting such forces, particularly in the low pressure range. Under pressure, with conditions of reduced viscosity, the chromosomes should move apart all the more rapidly and easily if such forces were involved. Furthermore, since Marsland's law relating pressure and viscosity expresses a logarithmic relationship, the effect should be most noticeable in the low pressure range. Obviously this is in direct disagreement with the present findings.

But what is the role of gel structure in anaphase movement? Certainly there are at least two separable structures to be considered—the traction fibers and the spindle mass.

Considering the traction fibers first, Cornman (1944) in a thought-provoking review comes to the conclusion that they are contractile structures and supply the

force for movement. However, Cornman ignores one major difficulty in his otherwise excellent analysis. No one has yet been able to demonstrate that traction fibers thicken as they shorten, although this would be expected if we were dealing with contractile bodies. The author has certainly seen no evidence of this in his own preparations, nor has he been able to observe the converse of any visible thinning when a traction fiber was extended all the way across the cell from one pole to the other. We, therefore, seem to require a different explanation.

It is the author's thought that Schrader (1932) was correct in regarding traction fibers as being no more than passively semi-elastic structures. This has been given excellent experimental foundation by Ris (1943) who has been able to measure directly anaphase movement in living cells (insect spermatogonia and spermatocytes). In some cases he has demonstrated that anaphase movement is very definitely a two step process. The first, relatively rapid movement can be explained as due to the release of elastic tension so that the traction fibers do actually shorten. The remaining movement is then due to the spindle mass, with the traction fibers serving simply as passive connections to the chromosomes. Lewis (1939) produced an accelerated motion picture of dividing fibroblasts *in vitro* which beautifully showed the same phenomenon, although he has not commented upon it.

A general hypothesis of anaphase movement can be advanced on the assumption that the traction fiber is anchored at one end to the chromosome, and along some of its length to the larger gelled mass of the spindle which, in turn, is in motion. Thus it is simply a more or less elastic connection from the spindle body to the chromosome—a rope, so to say, between the machine and the load. This interpretation forces our attention to the body of the spindle.

The analysis of anaphase movement by Belar (1929) does much to delimit the problem, even though we cannot accept his general hypothesis. He demonstrated that it was impossible to account for the total movement on the basis of simple swelling or elongation of the main spindle mass (or, more specifically, the *Stemmkörper*). There is, however, an obvious way to avoid the difficulties outlined by Belar (other than his own solution), and still be consistent with his findings and other knowledge.

It is proposed that motion and force may be imparted to the spindle mass by means of two phase transformations. The postulate supposes that gel material is added either in the interzonal region^{*} or along the greater part of the spindle, while a proportional solation occurs at the poles. Thus a material circulation is established, but a circulation by means of sol-gel-sol transformations rather than within a single phase. Actually a somewhat comparable idea has been proposed by Wassermann (1929 and 1939). Such an idea would be regarded by many as entirely too speculative, and not subject to either proof or disproof. The author, however, wishes to point out some comparable effects which are not likely to be known to most cytologists.

Dan *et al.* (1938 and 1940) discovered a remarkable phenomenon in dividing sea urchin eggs. After the furrow completes its intrusion, an entirely new region of gelled cortex is added in the center of the furrow region as the original cortical

^{*} Note that Schmidt (1939) did not find birefringence with polarized light in the mid-region of sea urchin egg spindles, and that Shimamura (1940) found this to be the "weak" region in centrifuging experiments upon *Lilium* PMC.

material backs out. Pease (1943) calculated that this *de novo* cortex came to cover about 11 per cent of the cell surface. This gel growth is obviously analogous to a system that could very well work within a spindle.

Since the advent of hydrostatic pressure techniques, it has also become clear that all sorts of other cell processes involving movement are dependent upon gel structure. Thus amoeboid movement, cyclosis, streaming, cytoplasmic division, the movement of pigment granules, and the pole cell nuclei of *Drosophila* eggs, and even sperm penetration both through the egg surface and also to their final central position all cease (reversibly) when the gel is liquefied. All of these movements depend upon the rather unexpected, and admittedly little understood, properties of protoplasmic gels. Obviously the gel rearranges itself, and is itself in motion (cf., the review of Marsland, 1942). No doubt gel-sol transformations are usually if not always involved along with the rearrangement. Thus we do find empirically a common denominator for all movements other than such specialized activities as muscle contraction and ciliary motion. The author believes that a general theory of anaphase movement is in sight, and that it will come from a better physico-chemical understanding of protoplasmic gel-sol systems.

SUMMARY

Hydrostatic pressures have been applied to *Tradescantia* pollen mother cells as a technique for studying the structure of division spindles and chromosomes and the mechanics of anaphase movement. The procedure has given pertinent information by virtue of the fact that increasing pressures progressively reduce gel rigidity. Sufficiently high pressure results in liquefaction. Yet the effects are reversible.

The spindle of the first meiotic division was but slightly affected by 4,000 lbs./in.² pressure, yet was mostly liquefied by 5,000 lbs. The spindle of the second meiotic division withstood about 2,000 lbs. more pressure. The somatic cells were even more resistant.

Condensed chromosomes were significantly softened by even 1,000 lbs./in.² pressure as indicated by an undue elongation of the kinetochore stalk. Fusion bridges became particularly obvious when 3,000 lbs. was applied. Significant shortening and rounding occurred at 4,000 lbs. Total fusion and rounding, indicating complete liquefaction of the matrix, did not occur until pressures of 15,000 lbs./in.² were applied. The fusion and rounding appeared to be a surface tension effect, and suggested the existence of a true interfacial membrane between condensed chromosome and cytoplasm. Not even these highest pressures, however, affected the uncondensed prophase chromosomes so that the effect of pressure was thought to be only upon the matrix material.

Chromosome movement was limited to those pressures which did not liquefy the spindle. The presence of fusion bridges, however, resulted in very abnormal movement.

After the release of high pressures, spindles re-formed. That these were *de novo* structures was indicated by their sometimes abnormal orientation, by the frequency of multipolar spindles, and by abnormalities in the course of traction fibers. Thus, the traction fibers of two homologous chromosomes might go to a single pole. Abnormalities made it seem likely that the growth of traction fibers was in a large measure independent of the growth of the body of the spindle. The direc-

tion of growth of the traction fiber was not specifically oriented until it reached the oriented bulk of the spindle.

Chromosome movement in recovery material was abnormal in that the fusion bridges persisted. Thus the chromosome matrix which had been liquefied, had become highly viscous once more. Under such circumstances, homologous chromosomes frequently went to a single pole, and the traction fiber to the other pole extended all the way across the cell. However, such traction fibers were not thinner than normal.

The outstanding conclusion is that a gel structure in the spindle is essential for anaphase movement. The traction fiber apparently serves as nothing more than a semi-elastic connection between the chromosome and the main mass of the spindle which, in turn, is in motion. It is suggested that motion and force is imparted by means of sol-gel-sol transformations, with gel being added to the central bulk of the spindle while a proportional solution goes on at the poles.

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THE COMPARATIVE DISTRIBUTION OF TWO CHROMATOPHOROTROPIC HORMONES (CDH AND CBLH) IN CRUSTACEAN NERVOUS SYSTEMS

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INTRODUCTION

It was demonstrated by Brown (1933) that sea-water extracts of the crustacean central nervous organs contained material having a definite and characteristic effect upon certain chromatophores of the body. The nervous organs were the only tissues of the body other than the eyestalks, with their included sinus glands, that yielded such a chromatophorotropically active substance, thus suggesting that the former possibly contained a source or sources of normal, color-changing hormonal material. In the shrimp, *Palaemonetes*, injection of extracts of the nervous system were shown to bring about a rapid blanching of dark-colored specimens through concentration of the red and yellow pigments within the chromatophores, an action similar to that which could be induced by extracts of the sinus gland of the eyestalk.

Similar activity of the nervous system was described by Hosoi (1934) for *Penaeus japonicus* and by Hanström (1937) for *Penacus brasiliensis*. Knowles (1939) found that extracts of the central nervous system of *Leander adspersus* caused concentration of the white pigment within that species. Concentration of white pigment by extracts of central nervous system was also reported for *Cambarus* by Brown and Meglitsch (1940) who worked with the chromatophores in isolated pieces of integument. Sinus gland extracts had an antagonistic action upon this pigment, thus proving that the sinus glands and nervous system did not yield exclusively identical chromatophorotropic substances.

Evidence that the central nervous organs contained sources of hormones normally involved in the adaptive color-changes of *Palaemonetes* was presented by Brown (1935) who found that any vigorous stimulation of the cut ends of the optic nerves in darkened eyestalkless specimens would induce a blanching characteristic of that following injection of extracts of central nervous organs. Koller (1930) had also observed comparable responses of eyestalkless *Crago* but did not at that time consider the central nervous organs to be a source of the active material.

More convincing evidence for the production of a normal chromatophorotropic hormone in the crustacean nervous system was presented by Brown and Ederstrom (1940). Their observations concerned the reactions of the particularly sensitive melanophores in the telson and uropods of the shrimp, *Crago*. Amputation of the eyestalks of a white-adapted animal brought about, within 3-6 minutes, a complete dispersion of black pigment in the melanophores giving the animal a "black-tailed" appearance. The condition persisted for about an hour whereupon the pigment returned to its former concentrated state, the latter condition typically lasting for several days. Brown and Ederstrom found that the black pigment could be caused

to disperse again by stimulation of the eyestubs or by the injection of extracts of the circumoesophageal connectives. Upon more extensive experimentation they concluded that the mid-region of the connectives, including the connective ganglia, contained the origin of the *Crago* tail-darkening hormone (CDH) involved here. The results of these investigators were confirmed and extended when Brown and Wulff (1941) gave evidence for a second chromatophorotropic principle within the central nervous system, namely a *Crago* body-lightening hormone (CBLH) by describing that strong stimulation of the eyestubs simultaneously darkened the telson and uropods and lightened the remainder of the body, an action duplicated by injection of extracts of the central nervous system as a whole. It was shown that these two actions were due to two separable principles in that injection of ethyl-alcohol extracts of the nervous system gave only body-lightening action, the tail-darkening principle remaining in the alcohol-insoluble residue, and, that mild stimulation of the eyestubs of eyestalkless animals produced both tail-darkening and body-darkening. Brown and Wulff speculated that CDH was, in the absence of CBLH, a general body-darkening principle. This hypothesis was more specifically set forth and given experimental support by Brown (1946) who clearly demonstrated the source of this darkening principle to lie, not in the circumoesophageal connectives proper, but in the minute tritocerebral commissure interconnecting the connectives immediately posterior to the oesophagus. Injection of sea-water extract of this commissure in various experiments produced in every case tail-darkening but various degrees of either body-lightening or body-darkening. The variable effects upon the body seemed reasonably explained in terms of varying concentrations of an antagonistic body-lightening principle.

In the following experiments a survey was made of the effects of sea-water extracts of the central nervous systems of thirteen species of higher crustaceans representing the *Isopoda*, *Natantia*, *Astacura*, *Anomura*, and *Brachyura* upon *Crago* color-change. The distribution of both the *Crago* tail-darkening hormone, CDH, and the *Crago* body-lightening hormone, CBLH, was considered. We have concerned ourselves primarily with the presence or absence of each substance within the central nervous systems and, when the hormones are present in a particular species, with a survey of the relative concentrations of the principles within the parts containing the hormone in question.

EXPERIMENTS AND RESULTS

The experiments to determine the distribution of CDH and CBLH were conducted in the following manner. Animals for use in assaying the concentration of active principles in extracts of nervous tissue were first prepared. The eyestalks of a number of *Crago septemspinus*, ranging from 3-6 cm. in length, were amputated by means of a sharp scalpel and the eyestubs cauterized with an electric cautery needle. No animals were used for assay-purposes until at least twelve hours following this operation, at which time they could best be described as possessing mottled black and white bodies and light telson and uropods (see Fig. 1A, control).

A relatively simple but effective method was used in the preparation of central-nervous-system extracts. The donor of the nervous tissue first had eyestalks removed and stubs cauterized in the same manner as described above for *Crago*. The dorsal portion of the exoskeleton was then cut away. After removing surrounding

viscera and muscles the nervous organs were removed under a dissecting microscope by carefully severing the nerves about the brain, thoracic and abdominal cords and gently lifting the entire system out of the animal. Particular caution was observed in the removal of the circumoesophageal connectives so as to prevent any damage to the tritocerebral commissure. The nervous system was then placed in a watchglass containing a small amount of sea-water and divided by means of a sharp scalpel into the desired portions which usually comprised brain, connectives, thoracic cord, and abdominal cord.

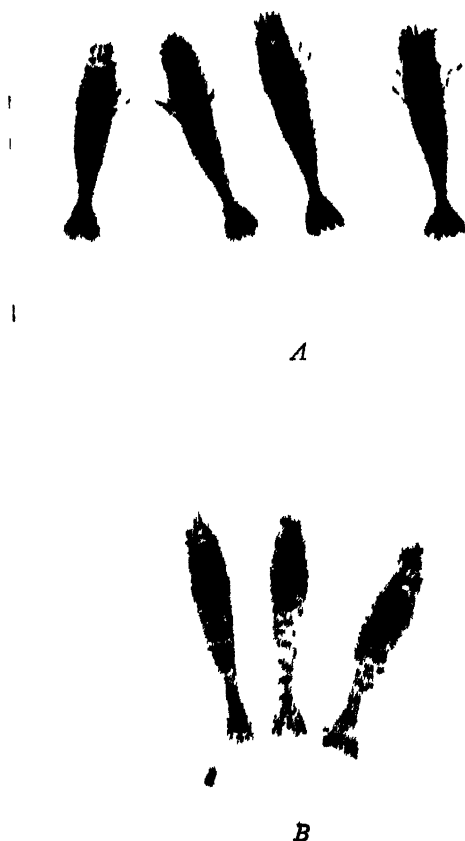


FIGURE 1. *A*. Darkening of eystalkless *Crago* following injection of a sea-water extract of the abdominal nerve cord of *Homarus* (conc. = 1 cord/0.5 ml. sea-water). The two specimens on the left are two uninjected ones used for a control. The injections for the animals on the right were made 15 min. before the photographs were made. *B*. Lightening of eystalkless *Crago* following injection of a sea-water extract of the circumoesophageal connectives of *Uca* (conc. = 3 pr. conn. to 0.2 ml. sea-water). The two specimens on the right were injected 8 minutes before the photographs were made.

Following this procedure the organs were transferred to individual glass mortars where excess sea-water was removed and the tissues allowed to dry partially. The tissue was then triturated with a measured amount of sea-water varying in quantity with the different species from 0.1–0.5 cc. per portion depending upon the size of the nervous system as a whole. In some cases, such as that of *Idothea*, it was necessary to use the parts of several nervous systems in the preparation of each extract in order to obtain adequate concentration and amount for assay. All extracts were centrifuged for three minutes at approximately 3,500 R.P.M. and the supernatant liquid of each injected into the dorsal musculature of the abdomen of at least two test-animals prepared as described above. The amount of extract injected into each varied with the size of the test-animal, but was normally between

TABLE I

Responses of eyestalkless Crago to injection of extracts of various portions of the central nervous system of other crustaceans. No. of cases signifies the number of donors

		Tail-darkening Time (min.)								Body-lightening ⊖ or darkening ⊕ Time (min.)							
Species	Organ	No. cases	0	5	10	15	30	45	60	0	5	10	15	30	45	60	
<i>Homarus</i>	Brain	7	0.0	3.3	3.6	3.9	3.7	3.7	1.6	0.0	0.0	+0.7	+2.0	+2.6	+1.9	+0.5	
	Connectives	8	0.0	1.9	2.3	2.3	2.3	2.1	1.4	0.0	-1.4	-1.0	0.0	+0.6	+0.4	0.0	
	Thoracic cord	8	0.0	1.6	2.1	2.2	2.8	2.7	2.5	0.0	-0.8	-0.3	+0.4	+2.8	+2.8		
	Abdominal cord	2	0.0	1.0	1.5	2.5	3.0	2.5	1.5	0.0	+3.0	+3.5	+4.0	+4.0	+4.0	+4.0	
<i>Cambarus</i>	Brain	10	0.0	3.4	3.4	3.4	2.6	1.3	0.4	0.0	+0.8	+1.2	+1.7	+0.9	+0.4	+0.2	
	Connectives	10	0.0	2.5	2.9	3.2	2.2	0.9	0.3	0.0	-1.7	-0.9	-0.6	-0.1	+0.1	+0.3	
	Thoracic cord	10	0.0	2.7	3.1	3.4	2.9	2.1	1.3	0.0	-1.9	-1.7	-0.9	+0.1	+0.3	0.0	
	Abdominal cord	10	0.0	2.6	2.8	2.8	2.5	1.4	1.0	0.0	+0.8	+1.2	+1.4	+1.1	+0.6	+0.2	
<i>Upogebia</i>	Brain	7	0.0	1.3	2.3	2.3	2.2	0.8	0.0	0.0	-2.0	-1.7	-1.5	-0.4	-0.2	0.0	
	Connectives	6	0.0	0.2	0.6	0.0	0.0	0.0	0.0	0.0	-2.3	-2.6	-2.0	-1.7	-0.4	-0.2	
	Thoracic cord	7	0.0	1.5	2.1	2.7	2.5	2.0	1.4	0.0	-0.2	+0.7	+1.2	+0.8	+0.5	+0.2	
	Abdominal cord	7	0.0	1.0	1.4	1.4	1.2	0.9	0.4	0.0	-0.6	-0.6	-0.3	0.0	0.0	0.0	
<i>Pagurus</i>	Brain	8	0.0	0.4	0.5	0.4	0.2	0.2	0.0	0.0	-1.6	-1.6	-1.1	-0.5	-0.1	0.0	
	Connectives	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.8	-1.6	-1.0	0.0	+0.1	+0.1	
	Thoracic cord	8	0.0	1.9	2.9	3.1	2.4	1.4	0.2	0.0	-1.3	-1.3	-1.3	-0.5	+0.2	0.0	
<i>Emerita</i>	Brain	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.5	-0.6	-0.5	-0.2	0.0	0.0	
	Connectives	8	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.4	-0.6	-0.2	0.0	0.0	0.0	
	Thoracic cord	8	0.0	1.0	1.6	1.6	1.0	0.5	0.1	0.0	-0.9	-0.6	-0.1	-0.1	0.0	0.0	
<i>Libinia</i>	Brain	7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-2.6	-2.8	-2.0	-1.0	-0.3	0.0	
	Connectives	7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.7	-1.8	-1.7	-0.4	-0.1	0.0	
	Thoracic cord	7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-1.7	-1.7	-1.8	-0.9	-0.4	0.0	

0.025 and 0.04 cc. Sea-water injected or uninjected controls were observed simultaneously with all test-animals.

Observations of the color changes in both body and tail were taken at five-minute intervals up to fifteen minutes and at fifteen-minute intervals thereafter. The degree of darkness of the tail or body was described within the range, +1 to +4, the number +4 representing the maximum extent of darkening and the number +1, the minimum observable one. In a similar manner body-lightening was indicated by the range, -1 to -4, with -4 denoting the greatest extent of body-lightening. Final results for a number of experiments were averaged and are presented in tabular form in Table I. These results have been further analyzed so as to present the distribution of CDH and CBLH within the central nervous system

of each of the species considered (see Tables II and III). In these tables, the relative distribution of activity of the hormones is calculated for the various portions of the nervous system for each species.

This was done as follows. The average values of the chromatophores at 5, 10, 15, and 30 minutes following extract-injection were of themselves averaged. Then for Table II the portion of the nervous system producing maximum darkening was

TABLE II

The quantitative distribution of CDH activity within the central nervous systems of a number of crustaceans. The region of maximum activity is arbitrarily given the value 1.00. It is important to note that each portion of the nervous system, regardless of size, is extracted in an equal volume of seawater, and the relative concentrations of the principles investigated are expressed solely in terms of their activities. This note applies equally to Table III.

Classification	Species or genus	Brain	Connectives	Thoracic cord	Abdominal cord
<i>Isopoda</i>	<i>Idothea baltica</i>	1.00	1.00	1.00	1.00
<i>Decapoda</i>		some			
<i>Natantia</i>	<i>Crago septemspinosus</i>	0.06	1.00	0.22	0.21
	<i>Palaemonetes vulgaris</i>	0.85	1.00	0.97	0.98
<i>Reptantia</i>					
<i>Astacura</i>	<i>Homarus americanus</i>	1.00	0.61	0.61	0.53
	<i>Cambarus virilis</i>	1.00	0.84	0.94	0.84
<i>Anomura</i>	<i>Upogebia affinis</i>	1.00	0.10	0.80	0.65
	<i>Pagurus</i> sp.	0.17	0	1.00	—
	<i>Emerita talpoidea</i>	0	0	1.00	—
<i>Brachyura</i>					
<i>Oxyrhyncha</i>	<i>Libinia</i> sp.	0	0	0	—
<i>Brachyrhyncha</i>	<i>Cancer irroratus</i>	0	0	0	—
	<i>Carcinides maenas</i>	0	0	0	—
	<i>Ovalipes ocellatus</i>	0	0	0	—
	<i>Uca pugilator</i>	0	0	0	—

arbitrarily given the value 1.00, the activity of the other parts being expressed in terms of simple proportions of this. For Table III the part showing maximum lightening was given the value — 1.00 with the activity of other parts similarly expressed proportionately. The positive values in the latter table obviously indicate darkening rather than lightening.

Within the single species of *Isopoda* investigated, *Idothea baltica*, there appears to be roughly a uniform distribution of CDH throughout the central nervous system, all organs darkening the telson and uropods of *Crago* to approximately the

same degree. Great variations in distribution of the hormones occur among the decapods. The Natantian, *Crago* apparently possesses significant CDH activity only in the regions of the circumoesophageal connectives. CDH is differentially distributed throughout the central nervous system of the anomurans with highest quantity usually in the posterior region of the thoracic cord, is relatively uniformly distributed within the central nervous system of the astacurans and *Palaemonetes*, and is entirely absent within that of the brachyurans.

The quantitative distribution of CBLH was considered here solely within the reptantian nervous system, although it is known to be present throughout the central nervous system of the natantians (Brown and Wulff, 1941). Both the anomurans and brachyurans show wide distribution of this principle throughout brain.

TABLE III

The quantitative distribution of CBLH activity within the central nervous systems of a number of crustaceans. The region of maximum body-lightening is arbitrarily assigned the value - 1.00. The + values indicate body-darkening.

Classification	Species or genus	Brain	Connectives	Thoracic cord	Abdominal cord
<i>Isopoda</i>	<i>Idothea baltica</i>	pres.	pres.	pres.	pres.
<i>Decapoda</i>					
<i>Natantia</i>	<i>Crago septemspinatus</i>	pres.	pres.	pres.	pres.
	<i>Palaemonetes vulgaris</i>	pres.	pres.	pres.	pres.
<i>Reptantia</i>					
<i>Astacura</i>	<i>Homarus americanus</i>	+2.40	-1.00	+2.20	+7.20
	<i>Cambarus virilis</i>	+1.09	-0.73	-1.00	+1.00
<i>Anomura</i>	<i>Upogebia affinis</i>	-0.64	-1.00	+0.27	-0.18
	<i>Pagurus</i> sp.	-0.92	-0.85	-1.00	
	<i>Emerita talpoidea</i>	-1.00	-0.86	-0.57	
<i>Brachyura</i>					
<i>Oxyrhyncha</i>	<i>Libinia</i> sp.	-1.00	-0.67	-0.71	
<i>Brachyrhyncha</i>	<i>Cancer irroratus</i>	pres.	pres.	pres.	
	<i>Carcinides maenas</i>	pres.	pres.	pres.	
	<i>Ovalipes ocellatus</i>	pres.	pres.	pres.	
	<i>Uca pugilator</i>	pres.	pres.	pres.	

connectives, and thoracic cord. However, a striking feature is noted in the astacurans and the natantian, *Palaemonetes*, in which a darkening (see Fig. 1A), as well as a lightening, of the body occurs.

The two species of astacurans with which we have concerned ourselves more or less parallel one another with respect to the distribution of CDH. In *Homarus* and *Cambarus* the region of greatest quantity of this principle is the brain, and is followed by an apparent gradual diminution of the substance from anterior to posterior within the nervous system. The problem of CBLH distribution seems somewhat more complex since, as has been previously mentioned, certain of these nervous-system extracts appear to produce body-darkening preceded by a body-lightening. The abdominal-cord extract is particularly active in body-darkening and only the

connectives and thoracic cords of *Homarus* and *Cambarus* show any body-lightening activity at all. In these cases where body-lightening is indicated, the lightening persists for only a short time and is followed by a definite darkening. These observations suggest that the body-darkening activity observable for extracts of the astacuran central nervous system is explainable in terms of CDH. It is significant that in no case is body-darkening ever obtained from a portion of the nervous system lacking tail-darkening activity. However, since there is no essential direct correlation between the degree of tail-darkening and the degree of body-darkening even within a single species, the observed results must be the consequences of varying proportions of the two principles within the extracts, with the degree of influence of either one being a function of its relative concentration at any given instant.

There are significant differences in the distribution of CDH within the group of anomurans. *Pagurus* and *Emerita* exhibit similar tail-darkening activities and these are shown chiefly by thoracic cord extracts. On the other hand, extracts of

TABLE IV

The responses of eyestalkless *Crago* to injections of extracts of parts of the thoracic cord of some anomurans, showing the differing distributions of CBLH and CDH activity. No. of cases signifies number of donors.

Tail-darkening Time (min.)										Body-lightening ⊖ or darkening ⊕ Time (min.)							
Species	Part of thor. cord	No. cases	0	5	10	15	30	45	60	0	5	10	15	30	45	60	
<i>Pagurus pollicaris</i>	Anterior $\frac{1}{4}$	8	0.0	0.3	0.3	0.4	0.3	0.0	0.0	0.0	-2.3	-2.2	-2.0	-0.6	-0.3	0.0	
	Second $\frac{1}{4}$	8	0.0	0.6	0.7	0.7	0.2	0.0	0.0	0.0	-0.7	-0.6	-0.3	-0.3	0.0	0.0	
	Third $\frac{1}{4}$	8	0.0	1.5	1.6	1.6	0.5	0.5	0.4	0.0	-0.4	-0.4	-0.3	0.0	0.0	0.0	
	Posterior $\frac{1}{4}$	8	0.0	2.6	2.6	2.4	0.9	0.5	0.0	0.0	-0.5	-0.5	-0.3	-0.1	0.0	0.0	
<i>Pagurus longicarpus</i>	Anterior $\frac{1}{4}$	6	0.0	0.5	0.5	0.5	0.0	0.0	0.0	0.0	-1.7	-1.6	-1.2	-0.4	-0.2	0.0	
	Second $\frac{1}{4}$	6	0.0	0.2	0.2	0.2	0.0	0.0	0.0	0.0	-1.0	-1.0	-0.3	0.0	0.0	0.0	
	Third $\frac{1}{4}$	6	0.0	1.3	1.4	1.5	0.8	0.5	0.2	0.0	-1.2	-0.8	-0.2	0.0	+0.2	+0.2	
	Posterior $\frac{1}{4}$	6	0.0	2.0	2.0	2.0	1.2	0.5	0.0	0.0	-0.5	-0.4	-0.2	-0.2	0.0	0.0	
<i>Emerita talpoides</i>	Anterior $\frac{1}{8}$	4	0.0	0.8	0.8	0.8	0.3	0.0	0.0	0.0	-2.3	-2.3	-1.8	-0.5	0.0	0.0	
	Second $\frac{1}{8}$	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	Third $\frac{1}{8}$	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.3	-0.3	0.0	0.0	0.0	0.0	
	Fourth $\frac{1}{8}$	4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	-0.5	-0.5	0.0	0.0	0.0	0.0	
	Posterior $\frac{1}{8}$	4	0.0	0.3	1.8	1.8	0.3	0.1	0.0	0.0	-0.3	+0.8	+0.8	0.0	0.0	0.0	

brain, thoracic and abdominal cords of *Upogebia* all contain notable amounts of CDH. Another similarity between extracts from *Pagurus* and *Emerita* is seen in the distribution of CBLH. CBLH is found in considerable amounts in brain, connectives, and thoracic cord of both genera. However, the thoracic cord extracts of *Upogebia* show almost a complete absence of CBLH activity while the extracts of the remaining parts of the central nervous system produce a definite body-lightening, the connectives being most active in this respect.

The absence of CDH within the brachyurans investigated as well as the restriction of this principle to the connective region of the natantians studied confirms the results of Brown and Ederstrom (1940). Experimental data show that moderate amounts of CBLH are found in brain, thoracic cord, and connectives. Although results for CBLH distribution for brachyurans are shown only for *Libinia*, it has been found that they are qualitatively the same for *Uca*, *Cancer*, *Carcinides*, and

Ovalipes. The striking body-lightening effect of a strong extract of *Uca* connectives and commissures is illustrated in Figure 1B.

An attempt was made to analyze further the localization of CDH and CBLH within the thoracic cords of *Emerita* and two species of *Pagurus*: *pollicaris* and *longicarpus* (Table IV). The procedure consisted of dividing the thoracic cords into a number of approximately equal portions, four in the case of *Pagurus* and five in that of *Emerita*. It was observed that the concentration of CDH within the thoracic cord of both *P. pollicaris* and *P. longicarpus* is greatest in the posterior fourth of the cord and decreases gradually along the cord as one proceeds anteriorly. In *Emerita* the highest region of CDH concentration is also the posterior portion of the thoracic cord. However, there is a lack of CDH in any of the central portions of the thoracic cord in *Emerita*. It would seem then that the distribution of CDH in the thoracic cord of *Emerita* is more restricted than in *Pagurus*.

The distribution of CBLH in the thoracic cord of *P. pollicaris* and *P. longicarpus* is similar. The most intense body-lightening effect is brought about by extracts of the anterior fourth of the cord while less intense reactions are produced by extracts of the remaining portions. Experiments with extracts of *Emerita* thoracic cord indicate a higher concentration of CBLH in the anterior portion of the cord, and apparent absence of CBLH in the second portion and only slight amounts of the principle in the third, fourth and fifth divisions of the cord. In summarizing the distribution of CDH and CBLH within the thoracic cords of *Pagurus* and *Emerita* we can say that CDH is relatively more concentrated posteriorly in the thoracic cord while CBLH appears more concentrated anteriorly.

DISCUSSION OF RESULTS

The effect of the extracts of the central nervous system upon the dark pigments of the telson and uropods of *Crago* possesses a characteristic pattern in each of the major groups of the order Decapoda. In the Natantian, *Crago*, we have observed the restriction of CDH activity to the circumoesophageal connectives, whereas the *Astacura* and *Palaemonetes* exhibit a more generalized occurrence of the hormone within the organs of the central nervous system. However, as one proceeds to the *Anomura*, these contain changes from the widespread condition in the astacurans to a more specialized one as evidenced by the restriction of CDH in the thoracic cord of two of the three genera examined. Finally there is an entire lack of CDH among the brachyurans.

Experimental data concerning the distribution of CBLH in the reptantians present an interesting problem. Although both the anomurans and brachyurans possess the body-lightening hormone in varying amounts throughout the entire central nervous system, the astacurans appear to limit the hormone to connectives and thoracic cord. The simplest explanation for the body-darkening activity of the astacuran central-nervous-system extracts involves action of the tail-darkening principle. It is thought that CDH produces body-darkening after CBLH has been exhausted or in the absence of CBLH. This is indicated in Figure 2 in which selected portions of the central nervous system of *Libinia*, *Cambarus*, and *Homarus* are shown to produce a graded series of differential effects upon the coloration of the body of eyestalkless *Crago*. These range all the way from maximum body-lightening and no trace of darkening (*Libinia* brain) through initial lightening followed by

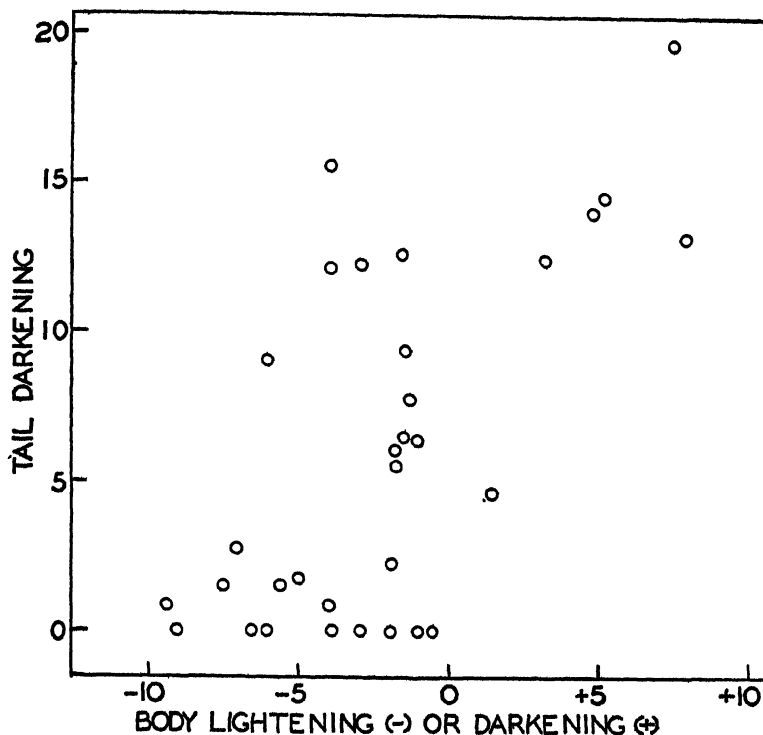


FIGURE 3. The general relationship between the degree of darkening or lightening of the body proper of eyestalkless *Crago* and the degree of darkening of the telson and uropods. Darkening of the tail is expressed as the algebraic sum of the intensities of the reactions at 5, 10, 15, 30, 45, and 60 min. following extract injection, thereby including a measure of both intensity and duration of the effect. Body-lightening, being more rapidly transitory, is expressed as the algebraic sum of the values at 5, 10, 15, and 30 min.

SUMMARY

1. A survey was made of the effects upon *Crago* color-change of sea-water extracts of various parts of the central nervous system of thirteen species of higher crustaceans. The crustaceans represented the groups *Isopoda*, *Natantia*, *Astacura*, *Anomura*, and *Brachyura*.

2. Extracts of various portions of the nervous system among the various groups showed wide differences in their total chromatophorotropic activities, producing various degrees of telson and uropod darkening and of body-lightening and darkening.

3. An analysis of the results gave support to the hypothesis that most crustacean nervous systems possess at least two principles, *a*) a *Crago* body-lightening principle, CBLH, lightening all portions of the body except telson and uropods, and *b*) a *Crago*-darkening hormone, CDH, darkening the telson and uropods, and, in the absence of CBLH, the body as well.

4. CBLH is more or less uniformly distributed throughout the nervous systems of all the species examined except the astacurans in which it is demonstrated only for the circumoesophageal connectives and thoracic cord.

5. CDH is restricted to the circumoesophageal connective region of the *Natantia*, is differentially distributed throughout the nervous systems of anomurans, with highest concentration in the posterior region of the thoracic cord, and is distributed throughout the nervous systems of the other species except the brachyurans in which it is absent.

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PHYSIOLOGICAL OBSERVATIONS ON WATER LOSS AND OXYGEN CONSUMPTION IN PERIPATUS¹

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The small group of species which comprises the Onychophora have long been of interest because of their unique combination of arthropod and annelid characters which places them in a phylogenetic position intermediate to those two extensive groups (Snodgrass, 1938). They are further of interest because of their close homogeneity despite a sporadic distribution that encompasses a large portion of the world and points to an ancient separation of some of the genera (Clark, 1915; Brues, 1923). This homogeneity appears to be physiological and ecological as well as morphological,² since *Peripatus* is restricted everywhere to a moist but terrestrial environment. Further, their sporadic and fluctuating local distribution suggests that environmental variation, presumably in moisture, is actively limiting their occurrence even in the regions where they are found.

Physiological observations on members of this group, then, are of interest, and it is of particular interest to examine the process of water loss and to contrast *Peripatus* in this respect to comparable annelids and arthropods. Manton and Ramsay (1937) have reported a value for water loss in *Peripatus* (*Peripatopsis*) at 30° and with wind velocity of 7.0 m./sec. (16 m.p.h.). These conditions, however, seem rather severe for a species which is uncomfortable at temperatures above 20° (Manton, 1938) and which, living in crevices, must have little exposure to wind. The experiments reported here were made under conditions which more nearly approximate those encountered by the animal in nature.

In this connection reports in the literature suggest that there may have been some temperature adaptation in the Onychophora. Thus the two species studied here, both from Panama (lat. 9° N.), stayed in good condition at a temperature of 25° ± 1. In contrast as already noted, *Peripatus* from near Cape Town (lat. 34° S.) became uneasy at temperatures above 20° although low temperatures, even down to freezing, did not bother them. They survived very well in England (Manton, 1938; Sedgwick, 1885) as have specimens from New Zealand (lat. 40° S.). The latter were only successfully transported through the intervening tropical regions with the aid of refrigeration (Sedgwick, 1887). *Peripatus* from New Zealand (Hutton, 1876) and from Australia (Steel, 1896) are reported to become torpid during the winter but with no subsequent ill effects. On the other hand Sclater (1887) reported that his specimens from British Guiana (lat. 7° N.) successfully

¹ These observations are by no means complete, but because the literature contains little data on living Onychophora, particularly on New World species, and because there was no immediate prospect of obtaining a further supply of these unusual animals, it seemed advisable to present them at this time.

² It should be noted, however, that for such a small group of Onychophora show remarkable diversity in their embryological development and their mode of reproduction.

survived the trip to England, "but unfortunately were much affected by the cold, and were therefore killed."

MATERIAL

These *Peripatus* were secured on Barro Colorado Island, Canal Zone, through the great kindness of Mr. James Zetek. Two species (note Clark and Zetek, 1946) were obtained, the larger of which (*Epiperipatus brasiliensis varians*) had a contracted length of 50 mm. and was uniformly colored a rich red-brown. The smaller species (*Oroperipatus corradi*) had a contracted length of 25 mm. and was a chocolate color with lighter underside and with darker legs and a dark, median, dorsal stripe 0.3 mm. in width. The animals were taken in early September and these observations were made in Cambridge about a month later. During the interim they were kept in moist forest debris but were not given suitable food other than the supply of termites initially in the debris. The animals survived the trip well and apparently stayed in good health until just before death which presumably occurred through starvation.

The general behavior of these individuals corresponded to that described for other species (Manton, 1938; Holliday, 1942; Andrews, 1933; Steel, 1896; Sedgwick, 1885; etc.). They were retiring and preferred to remain inactive in some dark crevice. They are sensitive to light but react even more sharply to dryness which stimulates them to constant activity. The smaller species were definitely more sensitive in this respect and could not be held still even for a moment.

An occurrence involving an individual of the larger species may be of particular interest. On the occasion of mechanical injury to one of its antennae that member was placed in the mouth and the injured portion, about half the length, was removed. The stump healed and the individual did not appear to be inconvenienced by the loss. Parturition as observed in these specimens has been described elsewhere (see Morrison, 1946).

The rate of oxygen consumption and water loss in *Peripatus* was compared to several arthropods and annelids of fairly similar size, habitat and body form: centipeds (*Lithobius*); millipeds (*Julus*); sow bugs (*Oniscus*); and earthworms. These were all collected locally with the exception of one small tropical earthworm found among the debris.

OBSERVATIONS

Sensory responses

With the exception of the antennae the animals showed equal tactile sensitivity all over the body, on the dorsal and ventral surfaces and on the legs. A very light stimulation could be applied with no response, a light one produced a local withdrawal of a leg or small section of the body, while a strong stimulus led to a general withdrawal. Holliday (1942) noted that fairly large wood lice and centipeds could crawl over the body of a *Peripatus* without evoking any response. The antennae are much more sensitive and the lightest touch here results in the retraction of one or both. With stronger or repeated stimulation the animal will completely contract and change its direction of progression; further irritation provoked the well known ejection from the slime glands. These responses are in accord with the histological findings of Manton (1937) that while a single well ensconced sense capsule was

found in each primary body papilla, each antennal papilla bore at least three much more exposed capsules with much heavier innervation.

The animals usually walked forwards but when startled would often reverse their direction, apparently walking backwards with equal ease. Occasionally they would half turn backwards and then move in the form of a "U" with the legs of the anterior half walking forward and those of the posterior half walking backwards. This mode of progression must impose an interesting problem in coordination.

The response of the animals to a single point source of light (a two-cell flashlight with reflector and glass removed, at a distance of 0.5 to 1 m.) was recorded by tracing their path on a large underlying sheet of paper. A number of records were made both with the light fixed and with it moved through 90 or 180° halfway through the record. Examination of the records showed no oriented negative phototropism; indeed, the animals actually travelled towards the light more often than away from it. Thus these animals would appear to be unable to localize light but only to be aware of it. This corresponds to the observations of Manton (1938) that the movement of objects near *Peripatus* elicited a response only when accompanied by air movement. These experiments were not carried out in a saturated environment, however, and it is possible that with the very strong stimulus of dryness removed, some phototropic pattern might be observed.

Water balance

In measuring water loss the animals were placed in large ($D = 5$ cm.) flat, weighing bottles containing a layer of calcium chloride covered by a floor of brass gauze. Measurements were made at 24° which is within the range normally encountered by these species (Kenoyer, 1929), and for periods of 30 minutes. No circulation was supplied, the movement of the animals themselves providing for convection. The *Peripatus* were particularly uneasy in this very dry atmosphere and kept in constant and vigorous motion.

The values obtained for the two species of *Peripatus* and for several other animals are summarized in Table I. Water loss has been computed on the basis of both body weight, and the two-thirds power of the body weight.³ The latter is perhaps a more reasonable basis for comparing animals of different size. The two values for *Peripatus* agree well and lie between those found for the annelids and arthropods. They indicate that *Peripatus* has a twofold advantage over the earthworm¹ in the conservation of water; and that it is at a twofold disadvantage as compared to the centipede, the most xerosensitive arthropod studied. Other arthropods showed values ranging down to one-twentieth that observed in *Peripatus*. These data are presented graphically in Figure 1.

Manton and Ramsay (1937) reported on water loss in *Peripatus* under the much more rigorous conditions of 30° with a 7 m./sec. (16 m.p.h.) wind and a rela-

³ This quantity is proportional to the surface area in animals of similar body form. In *Peripatus* and the arthropods where the actual body surface is increased by appendages and papillae, loss of water very probably takes place largely through the tracheae (note Mellanby, 1935). Water loss will therefore be related to respiration which is also roughly proportional to the two-thirds power of the body weight in animals of different size (Krogh, 1916).

⁴ This will be a minimum figure since the body weight of the earthworm includes a considerable amount of dirt in the gut. These earthworms were kept in clean wet containers for 1½ days before use, during which time they evacuated up to 15 per cent of their weight, but more undoubtedly remained.

tive humidity of 27.5 per cent. They found a value of 13.0 mg./g. min. or 2 to 3 times our value. A similarly measured value for an earthworm was about half as large on a weight basis or of equal magnitude on the basis of surface area. However, the advisability of making measurements under physiological conditions should be stressed since under abnormal circumstances quite different relations may hold. Thus, for example, Ransay (1935) showed that in the cockroach water was lost

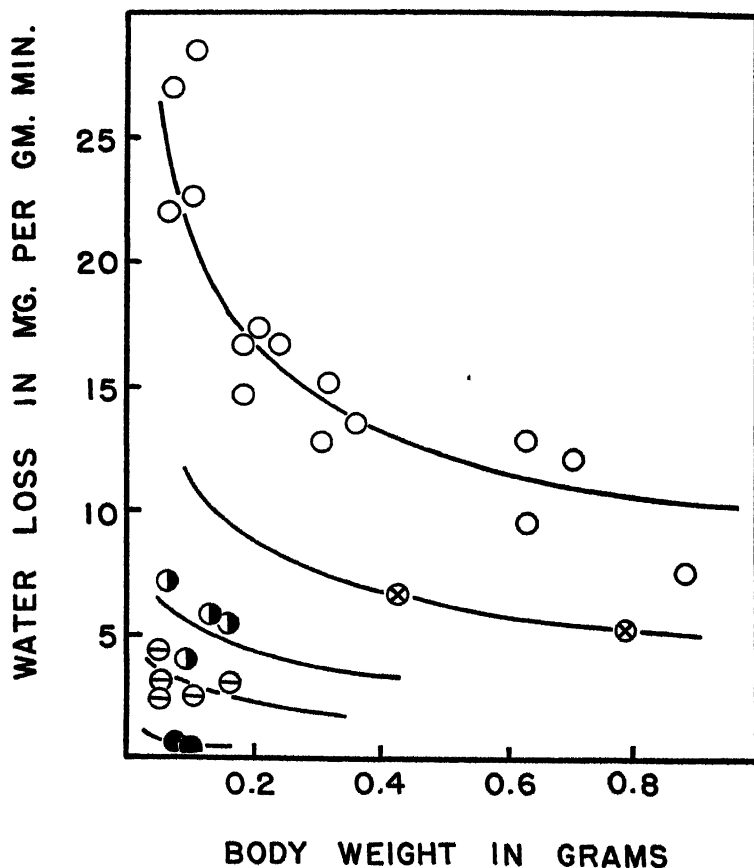


FIGURE 1. Water loss in *Peripatus* and other animals at 24° over calcium chloride as a function of the body weight. Open circles, earthworms; crossed circles, *Peripatus*; half-closed circles, centipeds; lined circles, sow bugs; closed circles, millipeds. The curves represent $Y = K(X)^{-0.75}$, where the values for K are the average values given in Table I.

much more rapidly at temperatures above 30° with an apparent breakdown of the hydrophobic character of the body surface.

In considering this function it is of interest to note that Clark (1915) concluded on the basis of distributional and taxonomic considerations that the Onychophora had originally evolved in a cooler rather than a warmer environment. Thus, the more primitive groups are found on mountains or in the "temperate" regions while

the more recent forms are tropical. This is, of course, entirely in accord with the physiological considerations since the xerotic stress would be reduced at a lower temperature and such an environment would be more favorable for evolution from an aquatic to a terrestrial mode of life.

TABLE I

Water loss in Peripatus and other animals at 24° over calcium chloride

Animal	Number and weight in mg	Duration of experi- ment in min	Water loss	
			mg /g.min.	mg /g ² /min
Earthworm	884	15	7.4	7.1
	703	15	12.0	10.4
	360	15	13.4	9.6
	208	10	17.3	10.4
	105	8	22.6	10.7
Peripatus	788	30	5.2	4.7
<i>Epiperipatus</i>	423	30	6.6	5.0
<i>Oroperipatus</i>				
Centipede	150	60	5.6	3.0
	135	20	5.9	3.1
	4×95	60	4.1	1.9
	4×63	30	7.2	2.8
Sow bug	158	25	3.0	1.6
	97	40	2.5	1.2
	6×49	60	3.0	1.1
	48	20	4.5	1.6
Millipede	3×76	120	0.56	0.24
	3×98	600	0.44	0.21
<i>Averages</i>				
Earthworm	15 Experiments			9.9
Peripatus	2 Experiments			4.9
Centipede	4 Experiments			2.5
Sow bug	5 Experiments			1.3
Millipede	2 Experiments			0.22

Respiration

The oxygen consumption of the larger species of *Peripatus* and of various other animals was measured in a Warburg apparatus.⁵ Carbon dioxide was absorbed in sodium hydroxide in a small cup fused to the bottom of the chamber. The animals were placed directly in the chamber and were kept from the lye by a small screen shield. Measurements were made at 25.0° C. over a period of 60 minutes.

The results on *Peripatus* are shown in Figure 2. After a restless initial period (10 minutes) it settled down to a very uniform rate of oxygen consumption. The centipeds, also shown in Figure 2, were less regular. The results for the various

⁵ I am indebted to Dr. William Carroll for the use of his calibrated Warburg assembly.

animals are summarized in Table II. The exact significance of the "resting" or "basal" oxygen consumption is not known but some correlation between it and the "intensity" of the organism has been observed. Compared on a weight basis *Peripatus* consumes oxygen at the same rate as the earthworm and at about half the rate of the arthropods. It has been observed, however, that within a given group, the metabolism per unit of weight varies with the size of the animal (note Edwards, 1946, for example), and that the metabolism is more nearly proportional to some

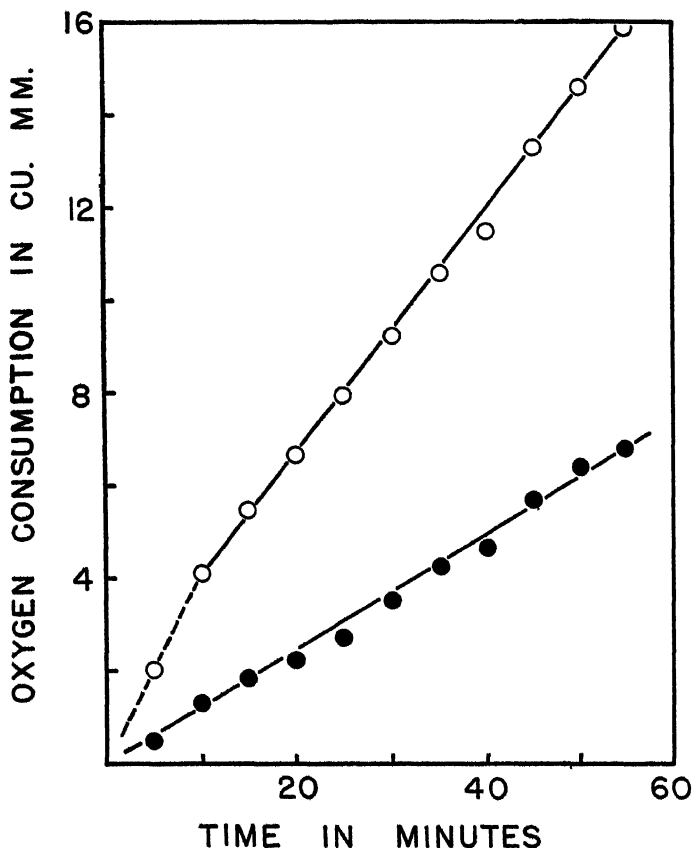


FIGURE 2. Oxygen consumption in *Peripatus* and the centipede as a function of time. Open circles, *Peripatus* (*Epipiripatus*), 0.68 g.; closed circles, 3 centipeds, total weight 0.33 g.; temperature, 25°.

lower power of the weight. As a first approximation this may be taken as the two-thirds power (Krogh, 1916). When the oxygen consumption is compared on this basis, *Peripatus* agrees more closely with the arthropods and has a higher value than the earthworm.

The hydrophobic character of the body surface has been noted by many observers. It is particularly evident when the animal is submerged since the body papillae hold the water away from the body surface and leave the animal entirely

surrounded by a sheath of air. It would seem entirely possible that this air sheath may function as a respiratory surface under water. Such a mechanism has been demonstrated in certain aquatic insects which carry down an air supply by means of hydrophobic hairs and which, by this means, greatly extend their periods under water (Krogh, 1941; Wigglesworth, 1931). Since *Peripatus* must be often covered by water in rainstorms, particularly as its lack of resistance to dessication forces it to frequent wet places, this mechanism could be of real utility and have a considerable survival value. This would provide a functional explanation for the papilla-covered body surface which is characteristic and unique in the Onychophora.

TABLE II
Oxygen consumption in Peripatus and other animals at 25° C.

Animal	Weight in mg.	Oxygen consumption	
		cc./g hr	cc./g ² /hr.
Earthworm	96	0.22	0.10 ¹
<i>Peripatus</i> (<i>Epiperipatus</i>)	680	0.23	0.20
Millipeds	3×111	0.46	0.22
Centipeds	2×69	0.56	0.22
Pill bugs	5×61	0.35 ²	0.14

¹ Lesser (1908) reported values of 0.4 cc. per g.²/hr. at 19° at which temperature the oxygen consumption should be about half that measured at 25° (Vernon, 1897).

² Edwards (1946) reports a similar value but at a temperature of 17°.

SUMMARY

The Onychophora represent a morphological transition between the annelids and the arthropods. They also represent a physiological transition between the aquatic and the terrestrial environment. In the latter transition the most important adaptations are those involving the functions of water conservation and respiration.

The ability of *Peripatus* to conserve water has been compared to that of comparable annelids and arthropods. *Peripatus* is shown to be intermediate to those two groups in this function, losing twice as much water as the centipede, but only one-half as much as the earthworm. This corresponds to its taxonomic and ecological positions.

The "resting" rate of oxygen consumption has also been compared to other animals. The rate in *Peripatus* is comparable to that in the arthropods and larger than that in the earthworm.

It is suggested that the unique papilla-covered body surface may represent an adaptation for underwater respiration to meet the environmental restriction imposed by the inadequate regulation of water loss.

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE
CHATTON AND LWOFF (ORDER HOLOTRICHA,
SUBORDER THIGMOTRICHA)

III. ANCISTROCOMA PELSENEERI CHATTON AND LWOFF,
ANCISTROCOMA DISSIMILIS SP. NOV., AND
HYPOCOMAGALMA PHOLADIDIS SP. NOV.

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INTRODUCTION

Chatton and Lwoff described in 1926 two ciliates for which they created the genus *Ancistrocoma*: *A. pelseneri*, from the gills and palps of *Macoma balthica* (L.); and *A. pholadis*, from *Barnea (Pholas) candida* (L.). Their descriptions of these two species are of a preliminary nature and are not accompanied by illustrations. More detailed descriptions of *A. pelseneri*, together with illustrations, are given in two papers of Raabe (1934, 1938).

Kofoed and Bush (1936) described as *Parachaenia myae* a ciliate from the pericardial cavity and excurrent siphon of *Mya arenaria* L., which Kirby (1941) noted was in several respects apparently identical with *A. pelseneri*. Kudo (1946), however, listed *Parachaenia myae* as a valid species in the suborder Gymnostomata. Kofoed and Bush stated that they did not find *P. myae* in any other molluscs which were present in the same localities as the host species. I have studied the ciliate associated with *Mya arenaria* in San Francisco Bay and have compared it with similar forms from *Cryptomya californica* (Conrad), *Macoma inconspicua* Broderip and Sowerby,¹ *Macoma nasuta* (Conrad), and *Macoma irus* (Hanley) from San Francisco Bay, and from *Macoma secta* (Conrad) from Tomales Bay, California. I have concluded that the ciliate described by Kofoed and Bush as *Parachaenia myae* is not specific in *Mya arenaria* and that *P. myae* is identical with *Ancistrocoma pelseneri* Chatton and Lwoff.

On the gills and palps of the rock-boring piddock *Pholadidea penita* (Conrad) there occurs a species of *Ancistrocoma* which is clearly distinct from *A. pelseneri* and which I will describe in this paper as *Ancistrocoma dissimilis* sp. nov. Another ciliate I have studied from *P. penita* is referable to the genus *Hypocomagalma*, created by Jarocki and Raabe (1932) for *H. dreissenae*, from the fresh water mussel *Dreissena polymorpha* (Pall.). It will be described herein as *Hypocomagalma pholadidis* sp. nov.

¹ By some malacologists the small species of *Macoma* referred to in this paper as *M. inconspicua* is considered to be conspecific with *M. balthica*; by others it is considered to be a subspecies of *M. balthica*. No conclusive evidence has been presented in the literature in recent years either to support or refute these contentions.

ANCISTROCOMA PELSENEERI CHATTON AND LWOFF

(Figure 1; Plate I, Figs. 1, 2)

The body is elongated and somewhat flattened dorso-ventrally.² As seen in lateral view, the ciliate is banana-shaped, the ventral surface being incurved. The anterior end is more or less attenuated. The body is usually widest and thickest in its posterior third. Forty living individuals taken at random from *Mya arenaria* ranged in length from $50\ \mu$ to $83\ \mu$, in width from $14\ \mu$ to $20\ \mu$, and in thickness from $11\ \mu$ to $16\ \mu$, averaging about $62\ \mu$ by $16\ \mu$ by $12.5\ \mu$. Twenty individuals from *Macoma inconspicua* ranged in length from $52\ \mu$ to $78\ \mu$, in width from $14\ \mu$ to $19\ \mu$, and in thickness from $11\ \mu$ to $15\ \mu$.

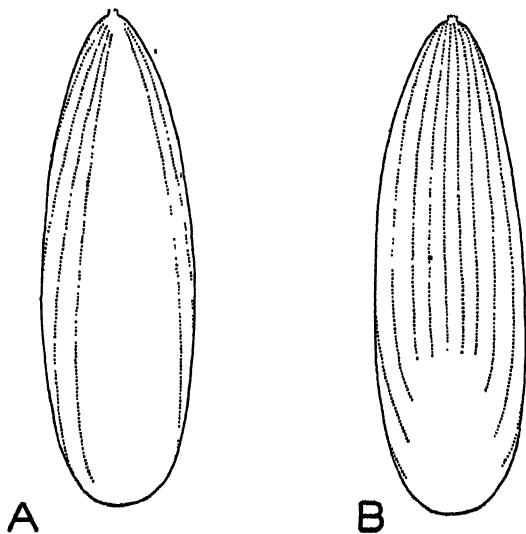


FIGURE 1. *Ancistrocoma pelseeneeri* Chatton and Lwoff. Distribution of ciliary rows, somewhat diagrammatic.³ A, dorsal aspect; B, ventral aspect.

The anterior end is provided with a contractile suckorial tentacle which enables the ciliate to attach itself to the epithelial cells of the gills and palps of the host and to suck out their contents. The internal tubular canal continuous with the tentacle is directed at first dorsally and then ventrally and obliquely toward the right side of the body. It can usually be traced in fixed individuals stained with iron hematoxylin for about two-thirds the length of the body. Kofoid and Bush suggested only in the title of their paper that the form which they named *Parachaenia myae* was parasitic in *Mya arenaria*, but did not describe attachment of the ciliate to the epithelium. They found the ciliate in the pericardial cavity and excurrent siphon

² Kofoid and Bush stated in their description of "*Parachaenia myae*" that the body of this ciliate is bilaterally compressed, the transverse diameter being about two-thirds the dorso-ventral diameter. Obviously their orientation of the form in question is not in agreement with the orientation assigned to it by Chatton and Lwoff, Raabe, and myself.

³ All text-figures illustrating this paper are based on camera lucida drawings of individuals fixed in Schaudinn's fluid and impregnated with activated silver albumose (protargol).

of the clams and apparently believed it to be unattached and to feed as a gymnostome, by producing a current in the medium by means of vigorous ciliary activity which carries food particles to the mouth. They stated that they observed a few instances of food taking, in which "debris containing bacteria enters the mouth and moves along the cytopharynx, forming little globules which continue back and aggregate in the large food vacuoles which distend the posterior part of the body." They stated further that "stained specimens show some vacuoles containing broken-up nuclear material similar to that of the epithelial cells which are removed when the fluid is taken from the clam." I have not observed any instances of ingestion of food such as that described by Kofoid and Bush, and although I admit it is perhaps possible for the ciliates to ingest food in this manner, I believe that they are primarily branchial parasites which feed by means of the suctorial tentacle.

The cilia of *A. pelsenecri* are disposed on the ventral, lateral, and dorso-lateral surfaces of the body in longitudinal rows originating at the anterior end. In all individuals which I examined carefully the number of ciliary rows was fourteen, but Raabe stated that in some specimens there are but thirteen rows. According to Raabe the ciliary system is composed of three separate complexes, the first consisting of eight or nine rows spiralling from the left side of the body toward the right and terminating progressively more posteriorly on the ventral surface, the second consisting of two approximately meridional rows situated on the central part of the ventral surface, and the third consisting of three rows spiralling from the right side of the body toward the left and terminating on the ventral surface. After studying a large number of the ciliates from *Mya arenaria* and *Macoma inconspicua* I cannot agree with Raabe on this matter. The ciliary rows appear collectively to form a single complex. There are usually five approximately equal rows about two-thirds the length of the body occupying the central portion of the ventral surface; these are bounded on the right by three progressively longer and more widely-spaced rows and on the left by six progressively longer and more widely-spaced rows. In some specimens the number of longer rows on the left side is greater than six, in which case the number of approximately equal and more or less meridional rows is proportionately decreased. Some of the outer rows on either side of the body, which originate on the lateral margins or on the dorsal surface, curve ventrally as they extend posteriorly, but the last two rows on the left side and the last row on the right side are typically dorso-lateral in position over their entire length. The outermost row on either side extends almost to the posterior tip of the body. Kofoid and Bush stated that the ciliary rows of "*Parachacnia myae*" may unite with one another, but I have never observed this to be the case, although in some seriously shrunken fixed individuals a few of the rows converge in such a way that they appear to be united.

In one of the illustrations accompanying the first of Raabe's papers in which there is a detailed discussion of *A. pelsenecri* (1934) the outermost ciliary row on the right side of the body is shown to originate as far anteriorly as the more central rows, while the outer three or four rows on the left side are shown to originate progressively more posteriorly. According to my own observations, however, the outermost row on the right side originates at about the same level as the last row on the left side. In all suitably impregnated individuals which I have studied the eighth row from the right side originates a little posterior to the level of origin of the adjacent ventral rows.

The cilia of *A. pelseneeri* are 8μ to 10μ in length. Those at the anterior end of the body are usually the more active and may be employed for thigmotactic attachment. Kofoed and Bush stated that the cilia of the "dorso-bilateral region" of "*Parachaenia myae*" are about 20μ long near the anterior end, becoming somewhat shorter posteriorly; the cilia of the ventral surface, on the other hand, were said by them to be about one-half the length of those of the dorso-bilateral area. I have noted, however, no significant disparity between the lengths of the cilia of various parts of the ciliary system. When dissociated from the host the ciliate swims energetically, rotating on its longitudinal axis or swaying from side to side.

In the original description of *A. pelseneeri* given by Chatton and Lwoff reference is made to a "frange peristomienne" which they supposed corresponded to the peristomal fringe of cilia in species of *Ancistruma*. In his paper of 1934, Raabe described a short (approximately 13μ long) row of basal granules lying in a dorsal anterior depression just above the anterior part of the internal tubular canal which he thought may represent the "frange peristomienne" described by Chatton and Lwoff. In his paper of 1938, however, Raabe stated that on certain of his preparations of this ciliate he could distinguish a row of basal granules such as he described in 1934, but did not refer to it as the peristomal fringe, and suggested that Chatton and Lwoff may have mistaken the stained outline of the internal tubular canal for a row of basal granules homologous with those of the peristomal fringe of ancistrunid ciliates. In my study of living, stained, and impregnated individuals of the ciliate I believe to be *A. pelseneeri* I have found no evidence whatever of a dorsal anterior depression or a row of basal granules such as that described by Raabe.

Kofoed and Bush described internal fibrillar structures, which they believed to represent elements of the neuromotor system, extending for a short distance posteriorly from an annular commissure ("cytostomal ring") around the "cytostome." One of the fibrils was said by them to pass along the internal tubular canal ("cytopharynx") to a slight thickening on the surface of the canal, then "towards the dorsal surface where it joins a relatively large granule which is closely associated with the mid-dorsal ciliary fibril." They stated further that "from points of the cytostomal ring on the ventral side, two fibrils are given off which soon unite and continue as a slender thread along the ventral surface of the cytopharynx." I have been unable to detect any structures in *A. pelseneeri* which might be construed as elements of a neuromotor system, but perhaps it is a siderophilic fibril-like structure of the type that Kofoed and Bush described that Raabe may have thought to represent a series of basal granules. The "cytostomal ring" around the "cytopharynx" was stated by Kofoed and Bush to be connected with the longitudinal ciliary rows,

EXPLANATION OF PLATE I

FIGURE 1. *Ancistrocoma pelseneeri* Chatton and Lwoff (from *Mya arenaria*). Ventral aspect. Heidenhain's "susa" fixative-iron hematoxylin. $\times 1,680$.

FIGURE 2. *Ancistrocoma pelseneeri* Chatton and Lwoff (from *Macoma inconspicua*). Lateral aspect from left side, from life.

FIGURE 3. *Ancistrocoma dissimilis* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,680$.

FIGURE 4. *Hypocomagalma pholadidis* sp. nov. Dorsal aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,260$.

FIGURE 5. *Hypocomagalma pholadidis* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,260$.



PLATE I

but I have not observed this to be the case in *A. pelseneeri*. As has been pointed out above, some of the rows do not originate as close to the base of the suctorial tentacle as others. It is possible that the structure referred to by Kofoid and Bush as the "cytostomal ring" represents the siderophilic anterior edge of the contracted suctorial tentacle.

The cytoplasm is colorless and contains numerous small refractile granules of a lipid substance. In the posterior part of the body there are in addition to typical food vacuoles containing ingested fragments of epithelial cells one or more large vacuoles containing globular masses usually of a dense, homogeneous character. Raabe referred to this type of vacuole as "Konkrementenvacuole" and suggested that since he observed the internal tubular canal to terminate very near the "Konkrementenvacuole" the material within the vacuole may represent an accumulation of waste material which was not digested and absorbed as the ingested food material passed backward down the canal. It is quite true that these concretment vacuoles do not resemble the typical food vacuoles of most other ancistrocomid ciliates which I have studied. It would be interesting to determine whether or not digestion and absorption take place in the internal tubular canal, and how the material in the concretment vacuole, if it represents undigested wastes, is gotten rid of by the ciliate.

The macronucleus is usually sausage-shaped, rarely ovoid, and typically is situated dorsally near the middle of the body. In some fixed specimens stained with iron hematoxylin the chromatin appears to be distributed in irregular masses scattered through the macronuclear material; in other iron hematoxylin preparations and in most specimens stained by the Feulgen reaction the chromatin is aggregated into a dense reticulum enclosing vacuole-like clear spaces. In twenty individuals from *Mya arenaria* fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from $11\ \mu$ to $16\ \mu$ and in width from $4\ \mu$ to $7\ \mu$.

The micronucleus is ovoid, fusiform, or sausage-shaped, and usually is seen to lie to the right of the macronucleus. In fixed and stained specimens the chromatin is ordinarily aggregated into granules. In twenty individuals from *Mya arenaria* fixed in Schaudinn's fluid and stained by the Feulgen reaction the micronucleus ranged in size from $1.2\ \mu$ by $3\ \mu$ to $2.1\ \mu$ by $3.2\ \mu$.

Ancistrocoma pelseneeri is very common in *Mya arenaria* in all localities in San Francisco Bay where I have collected this mollusc. I have found it to be present, although usually in smaller numbers, also in *Cryptomya californica*, *Macoma inconspicua*, *M. nasuta*, and *M. irus* from several localities in San Francisco Bay, and in *Macoma secta* from Tomales Bay. It is peculiar that this ciliate was not recorded by Raabe from *Mya arenaria* at the marine biological station at Hel. Raabe listed *Sphenophyra dosinia* Chatton and Lwoff, *Hypocomidium granum* Raabe, and a species of *Ancistrum* which he provisionally referred to *A. cyclidioides* (Issel), from *M. arenaria*. I have found *S. dosinia* in a small percentage of *M. arenaria* and in a fairly large percentage of *Cryptomya californica* from San Francisco Bay. I have also found in *M. arenaria* the ciliate thought by Raabe to be *A. cyclidioides*, but not *Hypocomidium granum*.

Ancistrocoma pelseneeri Chatton and Lwoff (= *Parachaenia myae* Kofoid and Bush)

Diagnosis: Length $50\ \mu$ – $83\ \mu$ (according to Kofoid and Bush $40\ \mu$ – $100\ \mu$), average about $62\ \mu$; width $14\ \mu$ – $20\ \mu$, average about $16\ \mu$; thickness $11\ \mu$ – $16\ \mu$, average

about $12.5\ \mu$. The ciliary rows are fourteen (according to Raabe thirteen or fourteen) in number and are distributed on the ventral, lateral, and dorso-lateral surfaces of the body. There are usually five approximately equal rows about two-thirds the length of the body on the ventral surface, bounded on the right by three progressively longer and more widely-spaced rows and on the left by six progressively longer and more widely-spaced rows. The outermost row on either side extends almost to the posterior tip of the body. The more central rows originate close to the base of the suctorial tentacle, while the several outer rows on either side originate progressively more posteriorly on the lateral margins and the dorsal surface. Some of these rows curve ventrally as they extend posteriorly, but the two outer rows on the left side and the outermost row on the right side are typically dorso-lateral in position over their entire length. The macronucleus is usually sausage-shaped. The micronucleus is ovoid, fusiform, or sausage-shaped. Parasitic on the epithelium of the gills and palps of *Macoma balthica* (L.) (Wimereux [Chatton and Lwoff]; Hel [Raabe]); *Macoma inconspicua* Broderip and Sowerby, *Macoma nasuta* (Conrad), *Macoma irus* (Hanley), *Cryptomya californica* (Conrad) (San Francisco Bay, California); *Macoma secta* (Conrad) (Tomaes Bay, California); *Mya arenaria* L. (Tomaes Bay [Kofoid and Bush]; San Francisco Bay).

ANCISTROCOMA DISSIMILIS SP. NOV.

(Figure 2; Plate I, Fig. 3)

The body is elongated, attenuated anteriorly, and somewhat flattened dorso-ventrally. The ciliary system, to be described presently, is disposed for the most part on the incurved and slightly concave ventral surface. The body is widest and thickest in its posterior third and rounded posteriorly. Twenty living individuals taken at random ranged in length from $33\ \mu$ to $51\ \mu$, in width from $10\ \mu$ to $14.5\ \mu$, and in thickness from $8\ \mu$ to $12\ \mu$, averaging about $44\ \mu$ by $13\ \mu$ by $10\ \mu$.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The canal is directed at first dorsally and then ventrally and obliquely toward the right side of the body. In fixed specimens stained with iron hematoxylin it can usually be traced posteriorly for about one-half the length of the body.

The cilia of *A. dissimilis* are $7\ \mu$ to $8\ \mu$ in length and are disposed in longitudinal rows originating at the anterior end. The typical number of ciliary rows is eleven, but specimens with twelve rows are not uncommon, and I have seen some with fourteen rows. There are usually five approximately equal rows about three-fifths the length of the body occupying the central portion of the ventral surface; these are bounded on either side by three progressively longer rows, the outermost rows being three-fourths to four-fifths the length of the body. In specimens having twelve ciliary rows there are four longer rows on the left side instead of three; in specimens having fourteen rows there are four longer rows on the right side and five longer rows on the left. In some cases, particularly if the number of ciliary rows exceeds eleven, the five central rows are of unequal length, becoming progressively longer from right to left. One or two of the outer rows on either side originate on the lateral margin or the dorsal surface, usually a short distance posterior to the level of origin of the other rows. These rows curve ventrally and inward as they extend posteriorly, so that at least their distal portions are visible in ventral view.

The cytoplasm is colorless and contains numerous small refractile granules of a lipoid substance in addition to food inclusions. One or more larger food vacuoles are usually present in the posterior part of the body. The contractile vacuole lies near the middle of the body and opens to the exterior on the ventral surface.

The macronucleus is ovoid and situated dorsally near the middle of the body. In fixed and stained preparations the outline of the macronucleus is nearly always very irregular and the chromatin appears to be aggregated into a dense reticulum enclosing vacuole-like clear spaces of varying size. In twenty individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the macronucleus ranged in length from $6.8\ \mu$ to $13.7\ \mu$ and in width from $5.4\ \mu$ to $7.2\ \mu$.

The micronucleus is typically ovoid, rarely spherical, and commonly is situated a short distance anterior to or to one side of the macronucleus. In fixed and stained

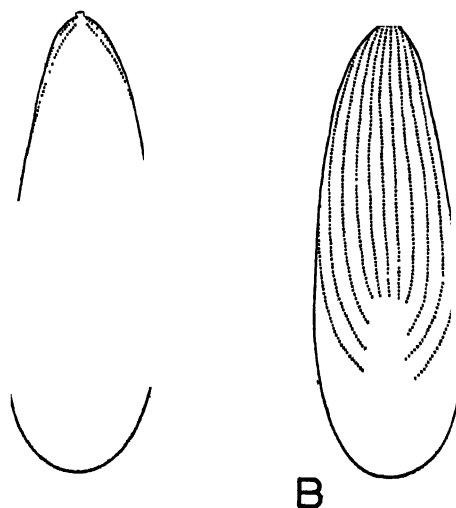


FIGURE 2. *Ancistrocoma dissimilis* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

preparations the chromatin appears to be dispersed in granules of varying size. In twenty individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the micronucleus ranged in size from $2.2\ \mu$ by $2.4\ \mu$ to $2.2\ \mu$ by $3.2\ \mu$.

I found *Ancistrocoma dissimilis* to be present on the gills and palps of twenty-one of thirty-six specimens of *Pholadidea penita* which I examined from localities near Moss Beach, California. It is sometimes found in association with *Hypocomagalma pholadidis*. In some individuals of *P. penita* I have encountered a ciliate of the genus *Sphenophrya* which I hope to describe in a later paper and a species of *Boveria* which may also be new.

Ancistrocoma dissimilis sp. nov.

Diagnosis: Length $33\ \mu$ – $51\ \mu$, average about $44\ \mu$; width $10\ \mu$ – $14.5\ \mu$, average about $13\ \mu$; thickness $8\ \mu$ – $12\ \mu$, average about $10\ \mu$. The ciliary rows are eleven to fourteen (typically eleven) in number and are distributed for the most part on the

ventral surface and lateral margins of the body. Most of the rows originate on the ventral surface close to the base of the suckorial tentacle, while one or two outer rows on either side originate on the lateral margin or the dorsal surface and curve ventrally and inward as they extend posteriorly. There are usually five approximately equal rows about three-fifths the length of the body bounded on the right by three progressively longer rows and on the left by four progressively longer rows. The outermost row on either side is three-fourths to four-fifths the length of the body. The macronucleus is ovoid. The micronucleus is typically ovoid. Parasitic on the gills and palps of *Pholadidea penita* (Conrad) (Moss Beach, California). Syntypes are in the collection of the author.

HYPOCOMAGALMA PHOLADIDIS SP. NOV.

(Figure 3; Plate I, Figs. 4, 5)

The body is elongated, strongly attenuated anteriorly, and markedly asymmetrical. The anterior end is deflected toward the left and bent ventrally. The dorso-ventral flattening characteristic of most ancistrocomid ciliates is not conspicuous in this species. As viewed from the posterior end the body appears in its middle and posterior portions to be almost as thick as wide. In its anterior third the body is nearly round in cross section. Most fixed specimens are considerably distorted and compressed in such a way that they appear to be widest near the middle. Twenty living individuals taken at random ranged in length from $63\ \mu$ to $89\ \mu$, in width from $18\ \mu$ to $25\ \mu$, and in thickness from $16\ \mu$ to $21\ \mu$, averaging about $76\ \mu$ by $22\ \mu$ by $19\ \mu$.

The anterior end is provided with a contractile suckorial tentacle continuous with an internal tubular canal. The canal can usually be traced in fixed specimens stained with iron hematoxylin down the middle of the attenuated anterior part of the body and then obliquely toward the right side. I have not succeeded in demonstrating the course of the canal beyond the anterior one-third of the body.

The cilia of *Hypocomagalma pholadidis* are approximately $9\ \mu$ to $10\ \mu$ in length. The ciliary system consists of twenty-four or twenty-five longitudinal rows. The body is almost completely invested by cilia except for a cilia-free "cap" at the posterior end. Two rows on the right side of the body usually appear to be set apart from the others, but in some specimens the spacing between these rows and the adjacent rows on either side is not significantly wider than the spacing between some of the other rows. Perhaps these two rows are homologous with the one or two rows constituting the right ciliary complex of *Crobricoma carinata* (Raabe), *Insignicoma venusta* Kozloff, and species of *Hypocomides*. They originate near the base of the suckorial tentacle on the right margin or the dorsal surface close to the right margin and curve ventrally and to the left as they extend backward. The outer row, as seen in ventral view, is the longer and extends almost to the posterior end of the body. The inner row terminates a short distance more anteriorly than the outer row, but is conspicuously longer than the first of the next series of rows, which usually is about two-thirds the length of the body. The first eight to ten rows to the left of the two longer rows all originate at about the same level on the ventral surface close to the base of the suckorial tentacle. The remaining rows, which are disposed along the left margin of the body and on the dorsal surface, originate progressively more posteriorly. The tenth or eleventh row of this complex is usually the longest, although some of the shorter rows on the dorsal surface

may terminate more posteriorly. The last ciliary row on the right side of the dorsal surface is always the shortest row, originating at a point about one-third the distance from the anterior end of the body to the posterior end and terminating at a point about three-fourths or four-fifths the distance from the anterior end to the posterior end.

The cytoplasm is colorless and contains numerous small refractile granules of a lipid substance in addition to food inclusions. One or more larger food vacuoles containing fragments of cells from the epithelial tissues of the gills or palps of the host are usually evident in the posterior part of the body. The contractile vacuole, when single, is located near the middle of the body and opens to the exterior on the ventral surface. In a larger percentage of the living specimens of *H. pholadidis* which I examined there were two or more contractile vacuoles scattered through

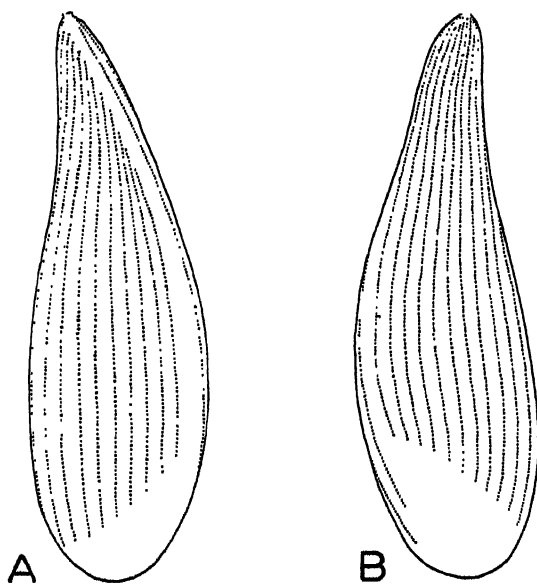


FIGURE 3. *Hypocomagalma pholadidis* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. A, dorsal aspect; B, ventral aspect.

the body which emptied their contents to the exterior on the ventral surface. In a large percentage of the living specimens of *H. pholadidis* which I examined there were two or more contractile vacuoles scattered through the body which emptied their contents to the exterior independently of one another. Jarocki and Raabe (1932) reported that in *H. dreissena* the contractile vacuole was sometimes single, but that in some specimens there were several smaller ones.

The macronucleus typically is sausage-shaped and lies in the posterior third of the body, its longitudinal axis placed obliquely to the longitudinal axis of the body. In light iron hematoxylin preparations and in specimens stained by the Feulgen reaction the chromatin of the macronucleus appears to be aggregated into a dense reticulum enclosing vacuole-like spaces which frequently contain globular masses of deeply-staining material. In ten individuals fixed in Schaudinn's fluid and

stained by the Feulgen reaction the macronucleus ranged in length from $12.5\ \mu$ to $20\ \mu$ and in width from $5\ \mu$ to $8.9\ \mu$.

The micronucleus is spherical and usually is situated a short distance anterior to or to one side of the macronucleus. In most fixed and stained preparations the chromatin appears to be homogeneous, although in some the chromatin appears to be in part aggregated into granules or peripheral strands. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the diameter of the micronucleus ranged from $2.4\ \mu$ to $3.3\ \mu$.

I found *Hypocomagalma pholadidis* to be present on the gills and palps of twenty-eight of thirty-six specimens of *Pholadidea penita* which I examined from localities near Moss Beach, California. When the ciliate is dissociated from the host it swims erratically, usually rotating on its longitudinal axis and tracing wide arcs with its attenuated anterior end. The cilia of the anterior half of the body are more active than those of the posterior half and are sometimes observed to beat metachronously. The ventral cilia near the base of the suckorial tentacle are markedly thigmotactic.

Hypocomagalma pholadidis sp. nov.

Diagnosis: Length $63\ \mu$ – $89\ \mu$, average about $76\ \mu$; width $18\ \mu$ – $25\ \mu$, average about $22\ \mu$; thickness $16\ \mu$ – $21\ \mu$, average about $19\ \mu$. The anterior end of the body is attenuated, conspicuously deflected toward the left, and bent ventrally. The ciliary system consists of twenty-four or twenty-five rows. Two long rows on the right side of the body appear in most specimens to be set apart from the remaining rows; these two rows originate near the base of the suckorial tentacle and extend almost to the posterior end of the body. The first eight to ten rows to the left of these two longer rows originate at about the same level on the ventral surface, while the remaining rows, disposed along the left lateral margin and the dorsal surface, originate progressively more posteriorly. The contractile vacuole may be single or represented by several independent vacuoles opening to the exterior on the ventral surface. The macronucleus is sausage-shaped. The micronucleus is spherical. Parasitic on the epithelium of the gills and palps of *Pholadidea penita* (Conrad) (Moss Beach, California). Syntypes are in the collection of the author.

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STUDIES ON CILIATES OF THE FAMILY ANCISTROCOMIDAE
CHATTON AND LWOFF (ORDER HOLOTRICHA,
SUBORDER THIGMOTRICHA)

IV. HETEROCINETA JANICKII JAROCKI, HETEROCINETA
GONIOBASIDIS SP. NOV., HETEROCINETA FLUMINI-
COLAE SP. NOV., AND ENERTHECOMA
PROPERANS JAROCKI

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INTRODUCTION

The genus *Heterocineta* was established by Mavrodiadi (1923) for a ciliate which he named *Heterocineta anodontae*, and which he had formerly believed to represent a gregariniform stage in the development of *Conchophthirus anodontae* (Ehrenberg). Unaware of the fact that Mavrodiadi had abandoned his earlier conception and applied the name *Heterocineta anodontae* to this ciliate, Jarocki and Raabe (1932) described the same species, from *Anodonta cygnea* (L.) and *Unio pictorum* L., as *Hypocomatophora unionidarum*. Jarocki later (1934) pointed out that *Hypocomatophora unionidarum* was a synonym of *Heterocineta anodontae*.

In his papers of 1934 and 1935 Jarocki described seven additional species of the genus *Heterocineta* ectoparasitic on fresh water gastropods: *H. janickii*, from *Physa fontinalis* (L.); *H. lwoffi*, from *Viviparus fasciatus* Müller; *H. chattoni*, from *Radix ovata* (Drap.); *H. krzysiki*, from *Bithynia tentaculata* (L.); *H. nasiarskii*, from *Coretus corneus* (L.); *H. turi*, from *Tropidiscus planorbis* (L.) and *Spiralina vortex* (L.); and *H. siedleckii*, from *Acroloxus lacustris* (L.). In 1945 I described as *Heterocineta phoronopsidis* a ciliate from the tentacles of *Phoronopsis viridis* Hilton. This species is the only representative of the genus thus far described which is not a parasite of fresh water molluscs or of the annelid commensal *Chaetogaster limnaci* von Baer when the latter is associated with infected snails.

On the fresh water prosobranch snails *Goniobasis plicifera silicula* (Gould) and *Fluminicola virens* (Lea) I have found two new species of *Heterocineta* which will be described herein as *H. goniobasidis* sp. nov. and *H. fluminicolae* sp. nov. I have also studied a species of *Heterocineta* from *Physa cooperi* Tryon which agrees with the original description of *H. janickii*. It seems advisable, for comparative purposes, and in view of the fact that Jarocki's description of *H. janickii* is not accompanied by illustrations, to include an account of the morphology of this form in the present paper.

The genus *Enerthecoma* was proposed by Jarocki (1935) for a single species, *E. properans*, parasitic on the gills of *Viviparus fasciatus*. Although the original description of this species is quite adequate, it is not supplemented by illustrations, and the second installment of Jarocki's "Studies on ciliates from fresh-water molluscs," in which figures of *E. properans* and several other ciliates were to be pub-

lished, has not come to my attention. A ciliate which I have found to infest *Viviparus malleatus* (Reeve) apparently is identical with *E. properans*. This ciliate will be described and illustrated here.

HETEROCINETA JANICKII JAROCKI

(Figure 1; Plate I, Fig. 1)

The body is elongated and flattened dorso-ventrally. The anterior end is attenuated, bent ventrally, and deflected slightly toward the left. The anterior one-half of the left margin is not quite so rounded as the right margin and typically is nearly straight or weakly indented. The body is widest a short distance behind the middle and rounded posteriorly. The ciliary system, to be described presently, is disposed on a shallow concavity occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. Twenty living individuals from *Physa cooperi* ranged in length from $25\ \mu$ to $32\ \mu$, in width from $12\ \mu$ to $15\ \mu$, and in thickness from $10\ \mu$ to $12\ \mu$,



FIGURE 1. *Heterocineta janickii* Jarocki. Distribution of ciliary rows, somewhat diagrammatic.¹ Ventral aspect.

averaging about $30\ \mu$ by $14\ \mu$ by $11\ \mu$. The specimens of *H. janickii* from *Physa fontinalis* which were studied by Jarocki ranged in length from $23\ \mu$ to $32\ \mu$, in width from $12\ \mu$ to $17\ \mu$, and in thickness from $10\ \mu$ to $13\ \mu$.

The anterior end of the body is provided with a short contractile suctorial tentacle which enables the ciliate to attach itself to the epithelial cells of the host and to feed upon their contents. When fully extended the tentacle is about $3\ \mu$ to $4\ \mu$ (according to Jarocki about $4.5\ \mu$) in length. The internal tubular canal continuous with the tentacle is directed at first dorsally and then ventrally and obliquely toward the right side, and in specimens stained with iron hematoxylin can usually be traced for about one-half the length of the body.

The ciliary system consists of eight longitudinal rows originating close to the base of the suctorial tentacle. The first four rows from the right side are approximately one-half the length of the body. The remaining four rows become increasingly longer and terminate one behind the other a little to the left of the midline.

¹ The text figures illustrating this paper are based on camera lucida drawings of specimens impregnated with silver nitrate by Klein's method.

The longest row is about two-thirds the length of the body. The cilia are about 6μ to 7μ (according to Jarocki about 5μ to 7μ) in length. While attached to the skin of the host the parasites are as a rule almost immobile, their cilia exhibiting only a feeble motion. When dissociated from the host *Heterocineta janickii* swims sluggishly, usually rotating on its longitudinal axis and tracing wide arcs with its attenuated anterior end.

The cytoplasm is colorless and contains numerous small refractile granules in addition to food inclusions. One or more large food vacuoles are present in the posterior part of the body behind the macronucleus. The contractile vacuole is situated near the middle of the body and opens to the exterior on the ventral surface. I have observed no permanent opening in the pellicle.

The macronucleus is typically sausage-shaped and is located near the middle of the body or somewhat posterior to the middle. As seen in dorsal or ventral view the longitudinal axis of the macronucleus is placed obliquely to the longitudinal axis of the body. As seen in lateral view, the anterior end of the macronucleus is directed dorsally, while the posterior end is directed ventrally. In fixed and stained preparations the chromatin appears to be more or less homogeneous. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 7μ to 11μ and in width from 4μ to 5μ .

The micronucleus is ovoid or spherical and is situated near the dorsal surface anterior to or to one side of the macronucleus. In most fixed and stained specimens the chromatin is homogeneous, although in some it appears to be concentrated in peripheral granules. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the size of the micronucleus ranged from 1.4μ by 1.4μ to 1.6μ by 2μ .

Heterocineta janickii was present in very small numbers on the tentacles, mantle, and margins of the foot of most of the specimens of *Physa cooperi* which I collected in a stream near Mt. Eden, California. The degree of infestation increased rapidly on snails kept in laboratory aquaria for a period of six weeks.

Heterocineta janickii Jarocki

Diagnosis: Length 25μ – 32μ (according to Jarocki 23μ – 32μ), average about 30μ ; width 12μ – 15μ (according to Jarocki 12μ – 17μ), average about 14μ ; thickness 10μ – 12μ (according to Jarocki 10μ – 13μ), average about 11μ . The ciliary system consists of eight rows originating close to the base of the suckorial tentacle. The first four rows from the right are about one-half the length of the body, while the remaining four rows become progressively longer and terminate one behind the other a little to the left of the midline. The longest row is about two-thirds the length of the body. Parasitic on the epithelium of the tentacles, mantle, and foot of *Physa fontinalis* (L.) (Warsaw [Jarocki]) and *Physa cooperi* Tryon (Mt. Eden, California).

HETEROCINETA GONIOBASIDIS SP. NOV.

(Figure 2; Plate I, Figs. 2, 3)

The body is elongated and flattened dorso-ventrally. The anterior end is attenuated, bent ventrally, and deflected slightly toward the left. The anterior one-half of the left margin is not so rounded as the right margin and typically is nearly

straight or weakly indented. The body is widest at the middle or a short distance anterior to the middle. The ciliary system is disposed on a shallow concavity occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. Twenty-five living specimens taken at random ranged in length from $36\ \mu$ to $48\ \mu$, in width from $15\ \mu$ to $20\ \mu$, and in thickness from $11\ \mu$ to $14\ \mu$, averaging about $43\ \mu$ by $18\ \mu$ by $13\ \mu$.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The nature of the canal is very similar to that of other members of the genus. It is directed at first dorsally and then ventrally and obliquely toward the right side of the body. It can be traced in most fixed specimens stained with iron hematoxylin for about one-half to two-thirds of the length of the body.



FIGURE 2. *Heterocincta goniobasidis* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. Ventral aspect.

The cilia of *H. goniobasidis* are about $9\ \mu$ long. Those of the anterior part of the ciliary system are markedly thigmotactic. The ciliary system consists of ten longitudinal rows. The first six rows are approximately the same length, being about one-half the length of the body, although on careful examination the first row is seen to originate some distance posterior to the level of origin of the other five rows. The seventh, eighth, ninth, and tenth rows originate progressively more posteriorly and become increasingly longer, terminating one behind the other a little to the left of the midline. The longest row is two-thirds to three-fourths the length of the body. The last one or two rows usually originate on the left margin and curve ventrally as they extend backward. The cilia of the distal portions of the longer rows are nearly always practically motionless and directed posteriorly. When dissociated from the host the ciliate swims sluggishly and erratically, rotating on its longitudinal axis.

The cytoplasm is colorless and contains numerous refractile granules of a lipid substance in addition to food inclusions. There are usually one or two large food vacuoles in the posterior part of the body behind the macronucleus. The contractile vacuole is central and opens to the exterior on the ventral surface.

The macronucleus is situated in the middle portion of the body. It is elongated and typically somewhat narrower at its anterior end than at its posterior end. As seen in dorsal or ventral aspect, the longitudinal axis of the macronucleus is placed obliquely to the longitudinal axis of the body. As seen in lateral view, the anterior end of the macronucleus is directed dorsally, while the posterior end is directed ventrally. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the macronucleus ranged in length from $10\ \mu$ to $13.5\ \mu$ and in width from $4\ \mu$ to $5.5\ \mu$.

The spherical or ovoid micronucleus is very difficult to distinguish in the living ciliates. It is usually situated near the dorsal surface a short distance anterior to the macronucleus. In fixed and stained preparations the micronucleus is vesicular, the chromatin being concentrated along the periphery. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the micronucleus ranged in size from $1.2\ \mu$ by $1.5\ \mu$ to $1.5\ \mu$ by $1.7\ \mu$.

Heterocineta goniobasidis was found to be present on the epithelium of the gills and mantle of a small percentage of the specimens of *Goniobasis plicifera silicula* which I collected in Crystal Springs Creek, in Portland, Oregon. The degree of infestation on freshly collected snails was very low, but increased during the four weeks the specimens were kept in laboratory aquaria.

Heterocineta goniobasidis sp. nov.

Diagnosis: Length $36\ \mu$ – $48\ \mu$, average about $43\ \mu$; width $15\ \mu$ – $20\ \mu$, average about $18\ \mu$; thickness $11\ \mu$ – $14\ \mu$, average about $13\ \mu$. The ciliary system is composed of ten rows. The first six rows from the right side are about one-half the length of the body and, with the exception of the first row, originate close to the base of the suctorial tentacle. The remaining rows originate progressively more posteriorly and become increasingly longer, terminating one behind the other a little to the left of the midline. The longest row is two-thirds to three-fourths the length of the body. Parasitic on the gills and mantle of *Goniobasis plicifera silicula* (Gould) (Portland, Oregon). Syntypes are in the collection of the author.

HETEROCINETA FLUMINICOLAE SP. NOV.

(Figure 3; Plate I, Fig. 4)

The body is elongated and flattened dorso-ventrally. The anterior end is attenuated, bent ventrally, and deflected slightly toward the left. The anterior part

EXPLANATION OF PLATE I

All figures except Figure 2 have been prepared with the aid of a camera lucida.

FIGURE 1. *Heterocineta janickii* Jarocki. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,720$.

FIGURE 2. *Heterocineta goniobasidis* sp. nov. Lateral aspect from left side, from life.

FIGURE 3. *Heterocineta goniobasidis* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,720$.

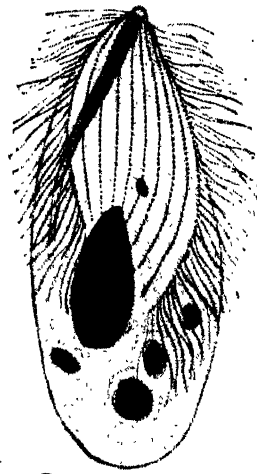
FIGURE 4. *Heterocineta fluminicola* sp. nov. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,720$.

FIGURE 5. *Enerthecoma properans* Jarocki. Macro- and micronuclei from three specimens. Schaudinn's fixative-Feulgen reaction. $\times 1,720$.

FIGURE 6. *Enerthecoma properans* Jarocki. Ventral aspect. Schaudinn's fixative-iron hematoxylin. $\times 1,720$.



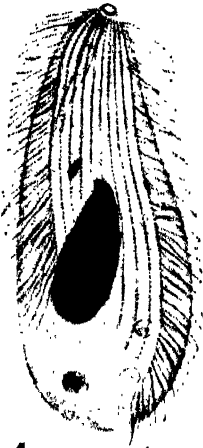
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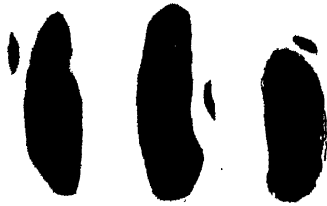
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5



6

of the left margin is not so rounded as the right margin and typically is weakly indented. The body is widest a short distance behind the middle and rounded posteriorly. The ciliary system is disposed on a shallow concavity occupying the major portion of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. Twenty-five living individuals taken at random ranged in length from $30\ \mu$ to $36\ \mu$, in width from $13\ \mu$ to $17\ \mu$, and in thickness from $10\ \mu$ to $12\ \mu$, averaging about $33\ \mu$ by $15\ \mu$ by $11\ \mu$.

The anterior end is provided with a contractile suctorial tentacle continuous with an internal tubular canal. The canal is directed at first ventrally and then obliquely toward the right side of the body. It can be traced in most fixed specimens stained with iron hematoxylin for about one-half the length of the body.

The cilia of *H. fluminicola* are about $6\ \mu$ or $7\ \mu$ long. Those of the anterior part of the ciliary system are strongly thigmotactic. The ciliary system consists of ten longitudinal rows. The first row on the right side of the ciliary complex origi-

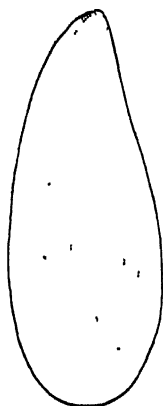


FIGURE 3. *Heterocincta fluminicola* sp. nov. Distribution of ciliary rows, somewhat diagrammatic. Ventral aspect.

nates close to the base of the suctorial tentacle; each of the remaining rows originates progressively more posteriorly. The first six rows from the right side are approximately the same length, being about two-thirds the length of the body. The last four rows become increasingly longer and incurved in such a way that they terminate one behind the other not far to the left of the midline. The longest row usually extends almost to the posterior end of the body. The cilia of the distal portions of these longer rows are usually directed posteriorly. When the ciliate is dissociated from the host it swims erratically, rotating on its longitudinal axis and tracing wide arcs with its anterior end.

The cytoplasm is colorless and contains numerous small refractile granules of a lipid substance in addition to food inclusions. One or more large food vacuoles are usually present in the posterior part of the body behind the macronucleus. The contractile vacuole is central and opens to the exterior on the ventral surface. I have not observed a permanent opening in the pellicle.

The sausage-shaped macronucleus is situated dorsally a short distance behind the middle of the body with its longitudinal axis placed obliquely to the longitudinal

axis of the body. In fixed and stained preparations the chromatin appears to be more or less homogeneous. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the macronucleus ranged in length from $7.4\ \mu$ to $10\ \mu$ and in width from $3.9\ \mu$ to $4.4\ \mu$.

The micronucleus is round, fusiform, or ovoid, and is usually placed dorsally near the middle of the body anterior to or to one side of the macronucleus. In fixed and stained specimens the chromatin is seen to be concentrated primarily along the periphery. In ten individuals fixed in Schaudinn's fluid and stained with iron hematoxylin the micronucleus ranged in size from $1.5\ \mu$ by $1.2\ \mu$ to $1.7\ \mu$ by $1.5\ \mu$.

Heterocineta fluminicola was present in small numbers on the epithelium of the gills and the edge of the mantle of nearly all specimens of *Fluminicola virens* which I collected in Crystal Springs Creek in Portland, Oregon.

Heterocineta fluminicola sp. nov.

Diagnosis: Length $30\text{--}36\ \mu$, average about $33\ \mu$; width $13\text{--}17\ \mu$, average about $15\ \mu$; thickness $10\text{--}12\ \mu$, average about $11\ \mu$. The ciliary system is composed of ten rows originating progressively more posteriorly from the right side to the left. The first six rows from the right side are about two-thirds the length of the body. The remaining four rows become increasingly longer and terminate one behind the other a little to the left of the midline. The longest row extends almost to the posterior end of the body. Parasitic on the gills and mantle of *Fluminicola virens* (Lea) (Portland, Oregon). Syntypes are in the collection of the author.

ENERTHECOMA PROPERANS JAROCKI

(Figure 4; Plate I, Figs. 5, 6)

The body is elongated, nearly symmetrical as seen in dorsal or ventral view, attenuated anteriorly, and flattened dorso-ventrally. The anterior end is bent ventrally and deflected inconspicuously toward the left. The ciliary system is disposed on a narrow, relatively flat area occupying the anterior two-thirds of the ventral surface; the dorsal surface and that part of the ventral surface posterior to the ciliary area are convex. The body is widest at a point about two-thirds the distance from the anterior end to the posterior end.* Twenty-five living individuals taken at random from *Viviparus malleatus* ranged in length from $32\ \mu$ to $56\ \mu$, in width from $13\ \mu$ to $21\ \mu$, and in thickness from $10\ \mu$ to $13\ \mu$, averaging about $44\ \mu$ by $18\ \mu$ by $11.5\ \mu$. Specimens from *Viviparus fasciatus* which were measured by Jarocki ranged in length from $33\ \mu$ to $60\ \mu$, in width from $15\ \mu$ to $22\ \mu$, and in thickness from $10\ \mu$ to $13\ \mu$.

The contractile suctorial tentacle is continuous with an internal tubular canal which is directed at first dorsally and then ventrally and obliquely toward the right side of the body. In specimens stained with iron hematoxylin the canal can usually be traced for about two-thirds or three-fourths the length of the body.

The ciliary system is composed of eight approximately equal rows about two-thirds the length of the body. These rows originate close to the base of the suctorial tentacle. The first five rows from the right side are usually a little more widely spaced than the last three rows. This was noted also by Jarocki, who stated that the ciliary system was separated into two complexes by an "inconsiderable eminence stretching from the base of the tentacle to the end of the system," which

segregated the five rows on the right from the three rows on the left. This eminence was evident on many of the living specimens which I examined but is never conspicuous. The cilia of *E. properans* are about 9μ in length and exhibit a feeble undulatory motion while the parasites are attached to the epithelium of the gills of the host. When dissociated from the host the ciliates swim slow and erratically, usually rotating on their longitudinal axes.

The cytoplasm is colorless and contains numerous small refractile granules of a lipoid substance in addition to food inclusions. One or more larger food vacuoles are usually present in the posterior part of the body. The contractile vacuole is situated a short distance behind the middle of the body and opens to the exterior on the ventral surface. I have not detected a permanent opening in the pellicle.

The macronucleus is typically sausage-shaped and is situated in the posterior half of the body with its longitudinal axis placed obliquely to the longitudinal axis

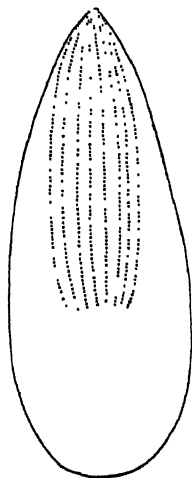


FIGURE 4. *Enerthecoma properans* Jarocki. Distribution of ciliary rows, somewhat diagrammatic. Ventral aspect.

of the body. In specimens stained with iron hematoxylin the chromatin appears to be more or less homogeneous, but in preparations stained by the Feulgen reaction it appears to be organized into a dense reticulum enclosing vacuole-like clear spaces of varying size. In ten individuals fixed in Schaudinn's fluid and stained by the Feulgen reaction the macronucleus ranged in length from 10μ to 19μ and in width from 4μ to 7μ .

The micronucleus is situated anterior to or to one side of the macronucleus. In most of the individuals of *E. properans* which I examined, the micronucleus is elongated and more or less fusiform. I have observed very few specimens to have a round micronucleus such as that described by Jarocki. The micronucleus does not stain readily with iron hematoxylin and it is possible that Jarocki may have mistaken food inclusions for micronuclei. In specimens stained by the Feulgen reaction the chromatin of the micronucleus appears to be concentrated in peripheral granules or strands. In ten individuals fixed in Schaudinn's solution and stained

by the Feulgen reaction the micronucleus ranged in size from $0.8\ \mu$ by $2.3\ \mu$ to $1\ \mu$ by $3.8\ \mu$.

Enerthecoma properans was abundant on the gills of nearly all specimens of *Viviparus malleatus* which I collected in Stow Lake, San Francisco, California, and in Evans Lake, Riverside, California. It is undoubtedly a common parasite of this introduced snail wherever the latter has become established.

Enerthecoma properans Jarocki

Diagnosis: Length $32\ \mu$ – $56\ \mu$ (according to Jarocki $33\ \mu$ – $60\ \mu$), average about $44\ \mu$; width $13\ \mu$ – $21\ \mu$ (according to Jarocki $15\ \mu$ – $22\ \mu$), average about $18\ \mu$; thickness $10\ \mu$ – $13\ \mu$, average about $11.5\ \mu$. The ciliary system is composed of eight approximately equal rows about two-thirds the length of the body which originate close to the base of the suckorial tentacle and occupy a narrow, relatively flat area on the ventral surface. The first five rows from the right are more widely-spaced than the remaining three rows, and in living specimens appear to be segregated from the latter by an inconspicuous longitudinal eminence. The macronucleus is elongated; the micronucleus is typically elongated and more or less fusiform (according to Jarocki, spherical). Parasitic on the gills of *Viviparus fasciatus* Müller (Warsaw [Jarocki]) and *Viviparus malleatus* (Reeve) (San Francisco, California; Riverside, California).

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PROGRAM AND ABSTRACTS OF SCIENTIFIC PAPERS PRESENTED
AT THE MARINE BIOLOGICAL LABORATORY, SUMMER OF 1946

JULY 9

DR. J. E. KINDRED. No abstract submitted.

The cyanide sensitivity of the unfertilized sea urchin egg. W. A. ROBBIE.

Reinvestigation of the cyanide sensitivity of unfertilized eggs of *Arbacia punctulata*, using recently devised methods for the control of HCN concentration in manometric experiments, showed that there was a definite inhibition of respiration. The respiration is depressed by concentrations of HCN as low as 10^{-5} M., and for a four-hour period in 10^{-4} M. it is only 40 per cent of the control value. There is complete inhibition for the first hour or more. In 4 per cent O₂-96 per cent N₂ mixture there is no depression of the respiration of the control egg, but on the addition of 10^{-4} M. HCN the oxygen consumption is reduced, for a four-hour exposure, to 20 per cent of the control level.

At concentrations of cyanide higher than 10^{-4} M. there is apparently a stimulation in oxygen uptake. This is increased with high and reduced with low oxygen tensions. It is possibly associated with oxidations proceeding through a cyanide-hemin system, or with the metabolism of a carbohydrate intermediate catalyzed by HCN.

Inhibition of fertilization in sea urchins by means of univalent antibodies vs. antifertilizin. ALBERT TYLER.

In order to obtain further information as to the role of the specific interacting substance of eggs and sperm in fertilization, antisera were prepared against them by immunization of rabbits, and the antibodies tested for their ability to interfere with fertilization. The present report concerns tests with antibodies prepared against purified antifertilizin derived from sperm of the sea urchin *Lytechinus pictus* and the gephyrean worm *Urechis caupo*. The antisera agglutinate the species sperm to high titer, but cannot be used directly to test for specific action on fertilization, since the mechanical effect of tying up the sperm would itself constitute a block to fertilization. However, by a previously described method, namely photo-oxidation, antibodies can be converted into a non-agglutinating form, termed "univalent." This treatment was, therefore, applied to the anti-antifertilizin sera and the "univalent" antibodies thus obtained were tested for possible action on the ability of the sperm to fertilize eggs of the same species. The results showed a considerable reduction in fertilizing power of the sperm, ranging in different tests from 32-fold to greater than 128-fold. At the same time, the motility of the treated sperm was found to be quite as high as the controls.

JULY 16

Intermediate steps in the visual cycle. A. F. BLISS.

The primary functions of a visual pigment are absorption of radiant energy and its transfer to the stimulatory mechanism of the visual cell. At present four such pigments are known: rhodopsin and porphyropsin, the photosensitive pigments of vertebrate night vision; iodopsin, the corresponding pigment of daylight vision; and cephalopsin, the photostable red pigment of cephalopods and probably other invertebrates (*J. Gen. Physiol.*, 1943). Instability in the light has generally been accepted as a diagnostic test for a visual pigment. The existence of a light-stable visual pigment in the squid however throws doubt on the validity of this assumption.

The bleaching by light of vertebrate visual pigments is nevertheless an interesting and complex process which has not been adequately analyzed into its component steps. The first known product of bleaching visual purple is a thermally unstable complex lipid, called Tran-

sient Orange by Lythgoe and Provisual Red by Krause. Its sluggish reaction to base and temperature near 0° C. suggest that it is the acid tautomer of the first relatively stable product of bleaching, appropriately named Indicator Yellow by Lythgoe. Acid Indicator Yellow is a red lipid, becoming reversibly decolorized in base, and irreversibly converted in chloroform to the greenish yellow carotenoid retinene, extracted by Wald from freshly bleached frog retinas. If bleached retinas are allowed to stand an hour before extraction, retinene is no longer found. Instead an equivalent amount of vitamin A is extracted. Retinene, however, is not the precursor to vitamin A, but is due to the irreversible side reaction described above. In the normal retina and in fresh neutral solution Indicator Yellow forms vitamin A under the influence of a labile protein, the reaction being presumably enzymatic in nature. In the dark, dissolved rhodopsin is reformed in part from Indicator Yellow. In the living animal the vitamin A released by bleaching is reincorporated into rhodopsin by unknown means.

MR. W. H. PRICE. No abstract submitted.

*The dependence of the resting potential of nerve on potassium, calcium, and hydrogen ions.** ABRAHAM M. SHANES.

On the basis of new as well as recent experimental results it now appears possible to describe a specific mechanism necessary and sufficient to account for the relationships between the resting potential and metabolic processes in frog nerve. Cellular hydrogen ion production accompanied by an exchange with extracellular potassium ions is apparently involved; under the conditions of study this process contributes about 50 per cent of the total resting potential. Calcium reduces the rate of ionic exchange, an effect of possible importance in the energy expenditure necessary to maintain concentration gradients and associated potentials.

The evidence consists of demonstrating first that hydrogen ions from (1) CO₂ produced by nerve, (2) CO₂ applied to nerve, and (3) lactic acid and possibly other sources of acid within the fibers are directly concerned with the production and maintenance of the potentials. This has been possible chiefly with the aid of inhibitors of carbonic anhydrase—sulfanilamide and thiophene-2-sulfonamide—by means of which the role of hydrogen ions can be followed during anoxia, upon return to oxygen following anoxia, and to some extent during relatively normal aerobic conditions. The changes of potential associated with the application of CO₂-O₂ mixtures and related experiments show that the effectiveness of the hydrogen ions is dependent on the ionic gradients established.

The involvement of extracellular potassium is demonstrated by the suppression in its absence of the changes in potential normally produced by CO₂. This effect is used to show, further, that the potassium in the extracellular spaces is reduced by the rapid large increase in potential induced by the return of the anoxic nerves to oxygen. A small secondary decline of potential which follows the rise in CO₂ and which is independent of extracellular buffering is also dependent on extracellular potassium, which suggests that the dense connective tissue or the other sheathing materials of nerve are interfering with the potassium exchange between the extracellular space immediately adjacent to the fibers and that more remote.

Calcium slows the rate of potential rise upon application of CO₂ and this effect is directly related to calcium concentration; in view of the above results and available evidence, this is interpreted as an effect on ionic exchange. At lower concentrations calcium depresses to almost the same degree the potential changes in response to oxygen following anoxia and to CO₂; higher calcium concentrations, known to suppress the metabolic processes, exert a more marked effect on the former.

These results therefore focus attention on factors important in the production and modification of the resting potential. The action of any agent on the potential must be considered from several possible standpoints: (1) inhibition or activation of metabolism or of carbonic anhydrase, (2) production of hydrogen ions, (3) production of a membrane diffusion potential, (4) modification of equilibrium or membrane diffusion potentials. The experimental procedures which have been applied provide means of distinguishing these possibilities. In view of the conclusions reached, these methods should also prove useful in studies of the "potassium pump" and of the biochemical processes concerned with CO₂ production and fixation in relatively intact cells, both are problems of considerable interest at the present time.

* Aided by a grant from the Penrose Fund of the American Philosophical Society.

DR. J T. BONNER. No abstract submitted.

JULY 23

Oxidation-reduction studies as a clue to the mechanism of fertilization of marine eggs. MATILDA M. BROOKS.

Eggs, sperm, and larvae at stages up to pluteus of three marine animals (*Arbacia punctulata*, *Asterias Forbesii* and *Chaetopterus pergmentaceus*) were measured for E_h and Ph. The egg, sperm or larvae were centrifuged and 1 cc. of the mass used for measurement in a glass vessel in the Coleman electrometer. It was found that there is a definite correlation between the rate of O_2 consumption and the redox potential of these cells. It was also found that the redox potential of sea water, as diluted by hypertonic NaCl, $CaCl_2$, $MgCl_2$, butyric acid or sucrose, became more negative than that of sea water alone. These facts were used as a basis for the hypothesis that a proper redox potential or ratio of oxidants to reductants of the respiratory enzymes was necessary for producing fertilization of the egg. The hypothesis as presented states that the redox potential of the external solution or sperm as compared with that of the exterior of the egg itself is an important factor in producing fertilization of the egg.

From the results with KCN in sea water which produced fertilization membranes but not cleavage, it was concluded that the formation of the fertilization membrane is not associated with oxidations, and appears rather to be due to change in the physical aggregation of some proteins at the surface of the egg or to a denaturation process occurring as the redox potentials is changing.

DR. C. L. YNTEMA. No abstract submitted.

JULY 30

The action of naphthoquinone antimalarials on respiratory systems. CHRISTIAN B. ANFISEN AND ERIC G. BALL.

In confirmation of the findings of Wendel (unpublished reports) a series of 2-hydroxy-3-alkyl-naphthoquinones have been found to exert a powerful inhibitory effect on the respiratory metabolism of the malarial parasite. The most powerful tested to date is the compound 2-hydroxy-3-(2-methyl-octyl)-naphthoquinone-1,4 (M-285) which, at a level of 1 mg./liter inhibits *Plasmodium knowlesi* respiration 60 per cent. In experiments to localize the site of action of M-285 in the main respiratory chain of enzymes it was found that p-phenylene-diamine oxidation, requiring only the cytochrome system, was not inhibited by the drug, while the oxidation of succinate to fumarate by succinic oxidase prepared from beef heart was completely inhibited at about 1 mg./liter. The drug, therefore, appears to inhibit at an oxidation-reduction potential level below that of cytochrome C. Succinic dehydrogenase activity, as measured by the Thunberg methylene blue technique, was only very slightly diminished even at high drug concentrations. Similarly, the respiration of both fertilized and unfertilized eggs of *Arbacia punctulata*, neither presumably containing succinic dehydrogenase, was inhibited strongly at levels as low as 0.1 mg./liter (2×10^{-7} M.). This enzyme, therefore, does not seem to be the inhibited system. A number of flavoproteins including d-amino acid oxidase and xanthine oxidase, as well as several systems involving the mediation of the pyridine nucleotides, showed no decrease in activity in the presence of the drug.

It appears possible that the naphthoquinones under study are inhibiting a hitherto undetected enzyme or enzyme group in the main chain of oxidative metabolism having an E_o below that of cytochrome C and above that of the flavoproteins.

Chemical sense and taste in the Sea Robin, Prionotus. ERNST SCHARRER.

The differentiation of taste and chemical sense is partly based on the concept that chemical sensitivity can evoke only negative or defensive reactions; positive reactions to food are mediated by the sense of taste (Kappers, Huber, Crosby, 1936, p. 347). Observations in the sea robin, *Prionotus*, do not support this conclusion. *Prionotus* possesses three free fin rays. Their

epithelium is innervated by spinal nerves; taste buds are absent. The afferent fibers end in accessory lobes on the dorsal surface of the cephalic end of the spinal cord. Secondary fibers from these lobes which represent the greatly enlarged dorsal horns, ascend cephalad to the funicular nucleus from which fibers pass ventromedially, crossing in the ventral commissure, and ending in the contralateral ventral horn. When the free fin rays of blinded and sufficiently hungry sea robins are stimulated with extracts of clams or crabs the animals react positively by turning to and snapping in the direction from where the juice comes. Positive reactions to chemical stimuli are mediated in this case by spinal nerves and in the absence of taste buds. The differentiation between chemical sense and taste can, therefore, be based only on the innervation and the presence or absence of taste buds. The reaction of the animal cannot be used as a criterion.

Studies of the respiration of the imaginal discs of Drosophila using the Cartesian diver ultramicrorespirometer. CLAUDE A. VILLEE.

A determination of the effects of a mutant gene on the metabolic activity of a particular group of cells provides a basic approach to the analysis of gene action in development. In most animals it is impossible to locate exactly the cells which will give rise to a particular structure, but in *Drosophila* each organ develops from a discrete group of cells, an imaginal disc, which can be dissected out of the larva. The rate of respiration of wing and leg discs from wild, "miniature" wing, and "vestigial" wing stocks were determined by the Cartesian diver ultramicrorespirometer and their weights measured by the quartz fiber balance of Lowry. The legs of the adults of all three stocks are normal, the wings of adult "miniature" flies are about two-thirds normal size but of normal shape, and the wings of adult "vestigial" flies are small misshapen stumps, less than one-quarter the size of the normal wing. At each of several stages studied, before, at and after pupation, the Q_{O_2} of wild type discs and the leg discs of all stocks used varied only slightly from 20 cu. mm. O_2 per hour per milligram of tissue. The Q_{O_2} of "miniature" wing discs was 18 and of "vestigial" wing discs 9 cu. mm. O_2 per hour per milligram of tissue. The weights of "vestigial," "miniature" and wild type wing discs are the same at corresponding developmental stages in the larvae and early (1-2 hour) pupae. The discs contain considerable reserves of substrate and will respire in the divers twelve hours or more. The mutant genes "vestigial" and "miniature" produce their effects by altering the rate of some chemical reaction in the wing disc of the larva which is reflected by a lowered rate of oxygen consumption. These results do not mean that the "miniature" and "vestigial" genes affect the same chemical reaction in development to a different extent but rather that in affecting different processes they each lower the overall metabolic rate of the disc. The metabolism of the leg discs, and probably of the other discs as well, is not changed, although the cells contain the mutant gene. The "vestigial" and "miniature" genes therefore produce their physiological as well as their morphological effects only in certain cells of the body, presumably due to the interaction of the gene or gene products with specific components of the cytoplasm of those cells.

AUGUST 6

The specificity of chlorine esterase. PHILIP B. ARMSTRONG.

The relative rates of hydrolysis of choline esters acting at the nerve terminations in the sphincter pupillae of the turtle could be inferred by determining the relative potentiations by eserine of threshold concentrations for pupillary constriction of the choline esters. A comparison of the potentiations *in vivo* with the relative hydrolysis rates of the choline esters by the purified specific choline esterase *in vitro* indicates that the enzyme as it functions *in vivo* is as specific if not more so than *in vitro*. The choline ester substrate concentrations *in vivo* at which eserine was effective were much lower than those for effective substrate hydrolysis *in vitro*.

DR. T. H. BULLOCK. No abstract submitted.

The endocrine role of the corpora allata in insects. BERTA SCHARRER.

In *Leucophaea maderae* (Orthoptera) extirpation of the corpora allata at nymphal stages earlier than the last causes an abbreviation of development (suppression of molts) which results in animals with adult-like characters ("adultoids"). In operated seventh instars the following nymphal molt is suppressed, and the animals emerge as adultoids, resembling normal adults except for their smaller size and comparatively shorter wings. Allatectomized sixth or fifth instars result in "pre-adultoid" stages which show less adultoid differentiation and require an additional molt before becoming adultoids. In the adult insect the corpora allata are necessary for the development of the eggs. In females allatectomized shortly after the beginning of a reproductive cycle the eggs do not develop appreciably beyond the stage typical of the ovary at the time of operation. The accessory sex glands in these operated females show little or no sign of secretion in contrast to normal control glands. Reimplantation of the corpora allata into allatectomized females causes the eggs and the nymphs hatching from them to develop as normally as those of unoperated animals. In a series of experiments in which the time of allatectomy is varied it can be demonstrated that the corpora allata are necessary throughout the period of growth and yolk deposition which constitutes about the first third of the total period required for the development of the eggs. The corpora allata are apparently not essential for the reproductive activity of male *Leucophaea*. Allatectomized males when mated with normal virgin females are capable of fertilizing the eggs.

Contrasts between visible and dominant lethal mutation rates in x-rayed Habrobracon eggs. ANNA R. WHITING AND H. C. GEORGE.

Senior author has previously reported that eggs x-rayed in late metaphase I have lethal dose about 2,000 r and one-hit dose-hatchability curve. Death appears to be due to terminal deletions. Eggs x-rayed in prophase I have lethal dose about 45,000 r and complex dose-hatchability curve. Death appears to be due to several factors, including translocations and inversions. Majority of lethal effects in both stages are dominant. Recently, two groups of females were treated with doses giving about 90 per cent mortality, one with 1,120 r for metaphase I and the other with 28,000 r for prophase I. They were then crossed with untreated males and their daughters were tested for heterozygosity for visible mutations. $\frac{F_1 \text{ } \text{♀♀} \text{ heterozygous}}{F_1 \text{ } \text{♀♀} \text{ tested}}$ was 2.11 per cent for eggs

treated in metaphase I, 12.69 per cent for eggs treated in prophase I. By χ^2 test there is less than one chance in one hundred that these stages belong to same class in respect to visible mutation rate although they have same dominant lethality rate. This strengthens theory of terminal deletions (which would not produce visibles) as most common response to x-rays of metaphase I. Visibles produced in metaphase I are probably genic and their low percentage is what would be expected at low doses tolerated by this stage. Most visibles from treated prophase I are probably also genic although a few may be due to position effects of translocations or inversions. Their high percentage is possible because of high doses tolerated.

AUGUST 13

A new factor from the adrenal influencing fat deposition in the liver. KATHERINE A. BROWNELL.

Starvation in the normal mouse leads to a large deposition of fat in the liver. This fails to occur after adrenalectomy. With these facts as a basis we have developed a test for a fat factor in various fractions prepared from ox adrenals.

The method is briefly as follows: Adrenalectomized mice are fed for 24 hours then fasted for 24 hours. During this 48-hour period they are injected every 6 hours with 0.2 cc. of the preparation to be tested. Two to 3 hours after the final injection the livers are removed and the total lipid determined gravimetrically.

Over 30 fractions from the adrenal gland including crystalline compounds have been tested by this method. The table shows results on adrenalectomized untreated animals, two fractions, a whole extract from which these fractions were taken and three crystalline compounds already

proven to have glyconeogenic potency. Both fractions are crude, being specific in only one respect—namely, that the carbohydrate factor fraction has no electrolyte potency and the sodium factor fraction no glyconeogenic potency. The only fraction that gave a highly significant response was that containing the carbohydrate factor. The low response given by whole extract, we attribute to inhibiting substances, three of which have been tested.

Since the liver fat response was given almost exclusively by the carbohydrate factor fraction, some of the crystalline compounds having glyconeogenic properties were tried to determine whether or not they were responsible. The table shows that the only one used which gave a significant response was dehydrocorticosterone; a 25 per cent increase over the control level and in order to obtain this response two and one half times as much pure substance (0.96 mgm.) was used as that estimated to be present in our carbohydrate factor fraction (0.35 mgm.). The other two compounds, corticosterone and 17-hydroxy-11-dehydrocorticosterone, gave liver fat responses only on the borderline of significance and to obtain even these small responses two to two and one half times as much material was used as that estimated to be present in the carbohydrate factor fraction. The fourth known glyconeogenic compound, hydroxycorticosterone, we were unable to test on account of lack of material.

There remain two possibilities: (1) that hydroxycorticosterone is the fat factor. If so, the effect on fat metabolism is a new property. (2) There is in the carbohydrate factor fraction a new factor regulating fat deposition in the liver.

Effect of adrenal fractions on deposition of fat in the liver

Treatment	No. of animals	Total lipid per cent	Increase per cent
Adept. untreated	29	6.31	—
Carbo. factor fraction *	15	8.42	33
No factor fraction *	7	6.74	9
Whole extract *	7	7.13	13
Dehydrocorticosterone †	8	7.87	25
Corticosterone †	8	7.11	13
17-hydroxy-11-dehydrocorticosterone †	7	6.87	9

* The extracts represent 300 gm. of tissue per cc.

† The solutions of crystals represent 0.6 mgm. solid per cc.

Hyperactivity of the adrenal cortex. FRANK A. HARTMAN.

At rest or under conditions of minimal activity there is a basal secretion of adrenal cortical hormones. In response to various stresses such as exercise, exposure to cold, trauma, anoxia, and poisons, there is an increase in output of the hormones which subsides after the stimulus disappears. After removal of a large proportion of both adrenals by enucleation, in the mouse, a considerable rise in the basal secretion occurs. This higher level of secretion is maintained for months. The following table illustrates these changes. Fat and glycogen (as sugar) in the liver were determined after 24 hours' starvation.

Values indicating changes in hormone production after enucleation of both adrenals

	Total lipid per cent	Glycogen per cent
Normal	8.5	0.12
Adrenalectomized	6.3	0.04
Enucleated 2 days	6.6	—
Enucleated 7 days	11.8	0.24
Enucleated 15 days	10.0	—
Enucleated 29 days	—	0.58
Enucleated 99 days	10.0	—

The wide difference in time at which the peaks for the production of the fat factor and carbohydrate factor occur, is evidence that the two factors are not identical.

By enucleation we removed an average of 75 per cent of the adrenal tissue. Less than 25 per cent of the active tissue remained since the circulation was disturbed and this 25 included the capsule. Thirteen days after enucleation the adrenals averaged 0.69 per cent of the body weight which is one-half the normal weight. Removal of cortical tissue probably reduces the inhibitory effect on the adrenotrophic hormone production by the pituitary so that after a lag of three or four days there is sufficient recovery of the remaining cortices to respond to the increased output of adrenotrophic hormone. However, the new level of cortical hormone production does not return the adrenotrophic output to the old level. Thus a higher basal level is established. The performance of a relatively small number of cortical cells indicates a large factor of safety. This capacity of cortical cells for sustained activity in disease where a large proportion of cortical tissue is destroyed is important in prolonging life.

There is now evidence for three mother hormones secreted by the adrenal cortex; the fat factor, the carbohydrate factor, and the sodium factor.

Studies on the mechanism of alloxan action. ARNOLD LAZAROW AND STANLEY LEVEY.

A number of compounds related to alloxan were synthesized and tested for their diabetogenic effect. These compounds were injected intraperitoneally into rats in high doses and the blood sugar was determined at 0, 1, 3, 8, 24, 48, and 72 hours after injection. Alloxan, N-methyl alloxan, and alloxantin which dissociates into alloxan all produced diabetes. N-N-dimethyl alloxan was toxic and, therefore, could not be injected in doses equivalent to that required for the production of diabetes with alloxan. Since alloxan is a ureid of mesoxalic acid, some derivatives were prepared in which the urea or mesoxalic acid portions of the molecule were intact. None of these (mesoxalamide, mesoxalic acid, dimethyl mesoxylate, or diacetyl urea) produced diabetes in the doses used. Freshly prepared dialuric acid, alloxanic acid, and barbituric acid did not produce diabetes; whereas, dialuric acid which was allowed to stand overnight was diabetogenic. (This is interpreted as oxidation of dialuric acid to alloxan by molecular oxygen.) Slight alterations in the structure of alloxan abolish its diabetogenic effect.

It has been reported by other investigators that alloxan combines with sulfhydryl groups of proteins and that on injection it produces a rapid drop in the blood and tissue glutathione. Since one of us has shown that injection of glutathione or cysteine immediately preceding a diabetogenic dose of alloxan completely protected the animals from diabetes; and since others have shown that pancreas contains less glutathione than do other tissues; it was suggested that variations in tissue glutathione may determine the selectivity of alloxan. Studies are now being carried out to determine the glutathione content of the beta cells of the pancreas which are selectively destroyed by alloxan.

Biological specificity and the synthesis of native proteins. DOROTHY WRINCH.

A common starting point for the discussion of biological specificity today is the assumption that biological function is an outward and visible sign of atomic pattern. Furthermore indications from many fields reinforce the old assumption that the native protein is the dominant structure type in all living systems. A vast number of physiological problems turn upon questions of atomic pattern, particularly such matters as (1) local stereochemical features and (2) the presence of internal $\text{OH}\cdots\text{O}$, $\text{NH}\cdots\text{O}$ and $\text{NH}\cdots\text{N}$ bridges and of linkages dependent upon the presence of a foreign ion.

Of all these problems, the most fundamental is the synthesis of native proteins. We must presume that the power of native proteins to produce replicas of themselves depends in some basic way upon their structure, and that it is intimately related to the presence on native protein surfaces of 'active patches' to use Warburg's term, each of which functioning as a template or mold permits the laying down on itself of a complementary constellation.

It is useful to notice that the associations of simple molecules within crystals offer many examples of such complementary constellations, e.g., (1) hexamethylene tetramine, with pairs which are not identical associated about tetrahedrally related planes with a common three-fold axis and (2) the phosphotungstic acid 29-hydrate with identical (i.e., self complementary) con-

stellations associated about such planes with a common three-fold axis and, in addition, self-complementary constellations associated about cube planes with a common two-fold axis.

Visualizing the formation of new 'active patches' on the surfaces of an already existing species of native protein molecules, we see that such new constellations comprise the material required for the formation of a new and identical molecule if (1) the species carries complementary patches (which may be but need not be individually self-complementary) and (2) the molecule is wholly made up of such patches, i.e., is a *surface structure*.

In order to have a mechanism whereby these isolated constellations on several different molecules may be integrated so as to interlock in the same spatial pattern as in the original molecule, something has to be postulated as to the capacity of the original molecules to form a crystal. Thus for example, let us visualize a body-centered cubic lattice with molecules placed at the 8 body-centers and the 6 cube corners nearest the origin, with the molecule at the origin missing. With the complementary constellations in position on each of the 8 + 6 faces of the molecules turned to the origin, we have a situation in which interlocking, possibly in a number of distinct steps, could take place, the resulting molecule being a replica of the original molecule. This is but one example of a number of such possibilities, with the original molecules characterized by antipodal pairs of complementary patches. All, however, have in common the dependence upon the capacity of the original molecules to crystallize, an outstanding characteristic and most remarkable property of unnumbered native proteins. Similarly, all theories as to the formation of new native protein molecules by autocatalysis must, it would seem, have in common the picture of such molecules as surface structures, i.e., atomic fabric cages.

AUGUST 20

Naturally occurring polyploidy in the development of Allium cepa L. Dr. C. A. BERGER.

One of the factors in the developmental pattern of *Allium cepa* is the formation of some tetraploid cells and their division as tetraploids. These cells are found throughout the cortex of the cotyledon and of the intermediate region between root and shoot. They are found in seedlings between 20 and 40 mm. in length. They are never found in the root. During prophase of mitosis in tetraploid cells the chromosomes are closely paired and relationally coiled. The two members of each pair are united at a single undivided SA-region. These cytological details show that the chromosomes have not separated since the time of their formation. Since the pairing and relational coiling is present from earliest prophase the double chromosome reduplication must have taken place during the resting stage immediately preceding the 4n division. At metaphase the tetrachromosomes undergo two successive divisions of the SA-region and anaphase is normal. Since no tetraploid division figures were found with unpaired chromosomes it was concluded that only one division of these tetraploid cells occurs.

Chick embryology at the medical schools of Ancient Greece. TAGE U. H. ELLINGER.

(Of the seventy titles comprising the Hippocratic Corpus, the most significant work, from a biological standpoint, is the lecture on embryology represented by the two texts *On Semen* and *On the Development of the Child*. It deals with human embryology from the formation of the semen to the birth of the child. The author is unknown, but he was not Hippocrates nor one of his followers. His work reflects the teachings of the medical school at Cnidos and of that of Empedocles whose influence is evident in doctrine as well as in scientific method and in the choice of vocabulary. This pre-Aristotelian author, who wrote in the last quarter of the fifth century B.C., at the time of Socrates, was indeed a very great scientist and a great teacher as well.

To the modern reader perhaps the most amazing revelation is the use made of observations on chick embryology in explaining to the students the development of the human embryo. The following quotations are in the author's translation.

In chapter 13, the Greek physician after describing a "semen which had stayed six days in the womb and which fell out," adds "A little later I will describe another test in addition to this one, that will enable anyone who seeks knowledge to see this for himself, as well as a proof that my whole discourse is correct, as far as that is possible for a mortal discussing such a matter."

He returns to this topic in chapter 29: "Now I shall recount the crucial test, that I promised a little while ago to make known, which is as clear as possible to a human intelligence and makes plain to anyone who wants to be informed about it, that the semen is in a membrane and that the navel is in the middle of it, and that it first draws air in and expels it outward," (according to the Empedocles' pneuma theory of differentiation) "and that there are membranes from the navel. You will also find the further growth of the child, as I have described it, to be from beginning to end, such as it is in my account, if you will apply the method of inquiry that I am about to describe. Take twenty eggs or more and give them to hatch to two hens or more; then on every day from the second to the last, that of hatching, remove an egg, break it and examine it. You will find that everything in it conforms with my statements, in so far as one can compare the growth of a bird with that of man. That there are membranes extending from the navel, and all my other statements about the child, you will find illustrated from beginning to end in the hen's egg; and he who has not yet made these observations will be surprised that there is a navel in a hen's egg. Such are the facts, and such is my account of them."

Again in chapter 30, the Greek author advances chick observations to illustrate and explain conditions in man. He states: "Now in proof of my theory, that it is the lack of nourishment that causes the child to come forth, provided it suffers no violence, I offer the following evidence. The bird develops from the yolk of the egg in the following way. Under the brooding mother the egg is heated and the content of matter inside receives the impulse to development from the mother. When the content of the egg is heated, it forms air and attracts other cold air from the atmosphere through the egg; for the egg is porous enough to admit the attracted air in sufficient quantity to the matter inside. The bird grows in the egg and is differentiated in the same or in a similar way to the child, as I have already said above. It develops from the yolk, but it receives its nourishment and material for growth from the white that is in the egg. This was at once apparent to all those who have given attention to it. Whenever nourishment from the egg is insufficient for the chick, then, not having sufficient nourishment to live on, it moves violently in the egg seeking more nourishment, and the membranes about it burst. When the mother notices that the chick has moved violently, she pecks and removes the shell. And this happens in twenty days. And it is evident that this is so, for, when the mother pecks the shell of the egg, there remains in it no liquid worth mentioning, since it has been expended on the chick."

Reproductive economy in closecrossed species with haploid males. P. W. WHITTING AND RUDOLPH G. SCHMEIDER.

According to the multiple-allele theory of sex determination, proved true for the wasp *Habrobracon*, every mating must involve either three or two sex alleles. The three-allele matings produce only females (sex heterozygotes) and normal (haploid) males (azygotes); but the two-allele matings produce also sex-homozygotes which either develop into sterile (diploid) males or are inviable. Outcrossing reduces the chance for two-allele crosses with their attendant reproductive wastage. The *Habrobracon* theory has been tentatively applied to the six or seven invertebrate groups characterized by male haploidy. Since many species, however, reproduce with much inbreeding, this theory would imply loss approximating half of the fertilized eggs. It has now been shown that in the wasp *Melittobia* over 90 per cent of the eggs from closecrosses, including selfcrosses (mother \times haploid son), may develop into females. If *Melittobia* females are sex-heterozygotes, some method must therefore have been evolved other than multiple alleles for avoiding production of sex-homozygotes equal in number to the females. Although the method of sex determination in *Melittobia* is not yet understood, it has now for the first time been shown that reproductive economy is high in a closecrossed species with haploid males.

A comparative study of the lipids in some marine annelides. CHARLES G. WILBER.

Studies on the metabolism of lipids have been in the past confined to observations made on vertebrates. Very few studies have been made on the lipids in the invertebrates; consequently a detailed investigation seems justified.

The following marine annelides were studied: *Nereis pelagica*, *Amphitrite ornata*, *Arenicola marina*, *Phascolosoma gouldii*, *Lepidonotus squamatus*, *Glycera americana*, and *Chaetopterus variopectatus*.

Whole worms or individual tissues were prepared by grinding or in the Waring-blendor. Lipids were extracted with boiling alcohol. Phospholipids were precipitated with acetone and magnesium chloride and estimated by oxidation-titration method of Bloor. Fatty acids were estimated by oxidation-titration and cholesterol colorimetrically using the acetic anhydride-sulfuric acid reagent. The ratios, cholesterol/fatty acid (lipocytic index) and cholesterol/phospholipid, were calculated.

It was found that the absolute values of the various lipids in the same species and in different species were not always the same. On the other hand, the lipocytic index and the relation, cholesterol/phospholipid, were constant for a given species and tissue. If the lipocytic index of each worm were plotted against the phospholipid of the same worm the points representing the various species fell along a straight line; a similar straight line was obtained when the cholesterol was plotted against phospholipid.

There is, therefore, an apparent relationship between cholesterol and phospholipid and between phospholipid and lipocytic index in marine annelides. Tissues with a high lipocytic index or high cholesterol content have a high phospholipid content. These results indicate that in the marine annelides, just as Bloor found in the vertebrates, since cholesterol is associated with and in constant relation to phospholipids, it is probably a normal protoplasmic constituent. These results confirm in part the results of analyses made on vertebrate tissues and agree with the contention of Mayer and Schaeffer that the lipocytic index is characteristic of the organ of an animal in a given species.

GENERAL SCIENTIFIC MEETINGS

AUGUST 23

Vascular reactions to ergonovine maleate as seen directly with the microscope in the living mammal.*¹ RICHARD G. ABELL.

Ergonovine was injected intravenously in amounts varying from 0.005 mgm. to 0.2 mgm., and its effect upon the arterioles, capillaries and venules observed directly with the microscope in transparent 'moat' chambers (Abell and Clark, '32) in rabbits' ears. The clinical intravenous dose of ergonovine is 0.1 mgm. The equivalent dose in the rabbit is approximately 0.005 mgm. Injections of 0.005 mgm. caused constriction of arterioles to approximately 0.7 to 0.9 of their control diameters, and a slight reduction in velocity of flow. The arterioles returned to their control diameters and the flow to its control rate within 3 to 5 minutes. Daily injections of 0.005 mgm. made for a period of 2 weeks caused similar results. One hundredth mgm. (twice the clinical dose) caused constriction of the arterioles to approximately 0.6 to 0.8 of their control diameters, and a slightly greater reduction in rate of flow than 0.005 mgm. One tenth mgm. (20 times the clinical dose) caused arterioles 15 to 30 microns in diameter to constrict to the point of obliterating their lumens and stopping the blood flow for approximately 30 seconds to one minute. The vessels relaxed to their control diameters within approximately 12 minutes. Two tenths mgm. (40 times the clinical dose) caused more vigorous and prolonged arteriolar constriction, which lasted for from 1 to 1½ minutes, and stopped all of the blood flow within the chamber. The venules constricted to approximately 0.6 to 0.7 of their control diameters. The arterioles returned to their control diameters in approximately 15 to 20 minutes. Four injections of 0.2 mgm. at 15 minute intervals made the small arterioles (15 to 30 microns in diameter) unresponsive to further injections, but not the larger arterioles (80 to 90 microns). Intravenous injections of 0.025 mgm. of epinephrine while the small arterioles were still unresponsive to ergonovine, caused them to constrict to the point of obliterating their lumens, which is the typical response to this amount of epinephrine.

None of the above injections caused any sign of injury to the blood vessels, or any abnormalities in appearance and distribution of the red blood cells, the white blood cells, or the platelets. Thus it is clear that ergonovine maleate, which is used widely to prevent post partum hemorrhage and to give symptomatic relief of migraine headache, does not cause any observable injury to the blood vessels and associated structures even when given in amounts of 40 times the clinical dose.

* "Ergotrate" (Ergonovine Maleate, U.S.P., Lilly).

¹ This work was aided by a grant made by Eli Lilly and Company to the Department of Anatomy of the University of Pennsylvania Medical School.

The effect of halogenated alkyl amines on the respiration of Arbacia eggs and sperm. E. S. GUZMAN BARRON, E. G. MENDES AND H. T. NARAHARA.

Halogenated alkyl amines at 0.001 M concentration produce an inhibition of the respiration of animal tissues, and complete inhibition of pyruvate and choline oxidation (Barron et al.¹). In smaller concentrations the early cleavage of the fertilized sea urchin egg is inhibited or retarded (Cannan et al.¹). There is also inhibition of mitosis in the corneal epithelium of mammals (Friedenwald and Scholz¹) and a high incidence of sex-linked lethals as well as a significant number of translocations and inversions in the chromosomes of *Drosophila melanogaster* (Auerbach et al.¹).

Dichloroethylmethylamine HCl, and trichloroethylamine HCl at a concentration of 0.001 M, and dissolved in sea water, produced a definite increase in the respiration of sea urchin sperm (from 170 to 50 per cent). The increase of respiration could be noticed even with 1×10^{-5} M. The respiration of sea urchin eggs, fertilized or unfertilized, was slightly inhibited by this concentration of alkyl amine (14 to 17 per cent). Higher concentrations produced inhibition of respiration probably due to a decrease in pII as a result of the hydrolysis of these compounds. When the alkyl amines were previously neutralized and the sperm and eggs suspended in 0.05 M citrate buffer, pH 6.8, the effect of the alkyl amines was erratic. It is quite possible that penetration of the alkyl amines into the cell occurs only in an acid milieu.

The experiments of Cannan et al.² on retardation of the rate of cleavage of fertilized *Arbacia* eggs were confirmed. Eggs treated with 0.001 M dichloroethylmethylamine HCl (dissolved in sea water) for 15 minutes prior to insemination, and fertilized eggs treated at the time of the first cleavage showed a definite retardation in the rate of cleavage. Furthermore none of the treated eggs reached the pluteus stage.

The effect of uranyl nitrate on the respiration of Arbacia sperm. D. BENEDICT AND E. S. G. BARRON.

Uranium, like other heavy metals, is quite toxic and it has been extensively used for the production and study of experimental nephritis. Uranyl nitrate in concentrations varying from 10^{-3} to 5×10^{-6} M. inhibited the respiration of *Arbacia* sperm. The inhibition was complete at 5×10^{-4} M. (92 per cent inhibition). 10^{-4} M. $\text{UO}_2(\text{NO}_3)_2$ produced partial inhibition (from 53 to 15 per cent), 5×10^{-5} M. inhibited 15 per cent, and 10^{-6} M. had no effect at all. This inhibition must be due to combination of respiratory enzymes with uranium, a combination which can be reversed completely on addition of a citrate at a ratio of U: citrate of 1:2. Addition of phosphate at a ratio of 1:100 brought only partial release (25 per cent). The experiments were performed in acetate-sea water buffer at pH 6.4 to avoid precipitation of the uranyl salt. Dry weights of sperm were obtained after centrifugation of the sperm at 16,000 g. There was in the control experiments a rise in the pH value of about 0.6 units at the end of one hour, probably due to the formation of NH_3 .

Some properties of purified squid visual pigment. ALFRED F. BLISS.

The photostable red visual pigment of the squid (Bliss, 1943, *Jour. Gen. Physiol.*) was found to become reversibly light sensitive in the presence of formalin. A method was devised for the extraction of this pigment in a state of purity approximating that of the best preparations of vertebrate rhodopsin. The principal impurity of previous extracts, melanoprotein, was rendered insoluble by the following procedure. Retinas were rinsed in distilled water and kept frozen until use. They were then homogenized with 0.2 M Na_2HPO_4 and centrifuged. The residue was washed with pH 4.5 buffer and distilled water. The visual pigment was extracted with 3 per cent digitonin at 6° C. for 2 minutes and centrifuged 5 minutes. The absorption spectrum of the extracted pigment did not differ significantly from that of rhodopsin. In its chemical properties it differed significantly from rhodopsin, since it was rapidly destroyed by digitonin even at 6° C. The primary breakdown product in cold acetone was, like that of rhodopsin (Bliss, 1946, *Biol. Bull.*), the acid tautomer of the lipid "Indicator Yellow." Because of the distinctive properties of the squid rhodopsin, a differentiating name, cephalopsin, is suggested.

¹ All quoted from Gilman, A., and Philips, F., *Science* 103: 409 (1946).

Studies on the viscosity and elasticity of striated muscle. MANFRED BRUST.

By the use of a spring vibrating against the resistance of frogs' (*Rana pipiens*) sartorius muscles—as described by Gasser and Hill (*Proc. Roy. Soc. B.* 96: 398, 1924)—the effects of urea and iodoacetic acid (IAA) on the viscosity and elasticity of these muscles were studied. All initial slack was removed from the system by stretching the muscles 17.5 per cent beyond their resting length and putting them under 3.5 gm. tension.

Thirty minutes immersion in solutions of 2.5 M urea in Ringer's shortens the muscles on the average by 26.1 per cent. When extended to their original length they still exert the tension originally exerted at that length. They will not return to their urea induced length when released from stretch. Their viscosity is reduced on the average of 53.6 ± 13.0 per cent of that in the untreated muscles, while the elasticity is similarly reduced to 60.4 ± 18.6 per cent.

Sixty minutes immersion of Ringer's equilibrated muscles in $1-80\,000$ IAA (6.72×10^{-4} M) in Ringer's sometimes causes a rise in viscosity and elasticity even without activity by the poisoned muscles. Summer frogs show this response less often than winter frogs. Measurements made during 30 second rest periods between 5 second isometric tetani show a short initial decrease followed by a gradual increase in both viscosity and elasticity. Average maximum rigor values of 181 per cent and 258 per cent respectively of the untreated muscle values are attained.

The urea results would agree with the findings by other authors that this agent disrupts myosin and other protein molecules thus transforming them into disconnected less asymmetric entities. Collagen is not believed to be markedly affected since muscle shape is maintained while tension remains the same as before treatment at the same lengths. The IAA results would agree with the progressively diminishing solubility and increase in hardness of actomyosin in gradually decreasing concentrations of adenosine triphosphate reported by the Szent-Gyorgyi group (*Acta Physiol. Scand.* 9: Suppl. xxv, 1945).

Arterial anastomoses. ELIOT R. CLARK AND ELEANOR LINTON CLARK.

This study represents an attempt to discover factors responsible for the presence or absence of arterial anastomoses, which vary so greatly in different organs.

The governing factor appears to be the histo-mechanical principle established by R. Thoma in 1892, corroborated by E. R. Clark in 1918 in studies on living vessels in the tadpole's tail, that the size of the lumen of an artery is regulated by the amount of blood flow. In the absence of flow, the lumen is reduced to zero and the artery obliterated. In order, then, for arterial anastomoses to survive, conditions must be such as to provide a flow of blood through the terminal connecting portion.

In most cases this requires the presence of factors which force the blood to flow part of the time in one and part of the time in the reverse direction. Such factors are present in the peripheral parts of the body in the form of varying outside pressures that are exerted irregularly upon large supplying arteries or small distributing arterioles.

A study, with the aid of artificial chambers, of the living circulation in the rabbit's ear, where anastomoses are abundant, reveals frequent reversals of flow in connecting portions of anastomoses, but controlled by an unsuspected factor, namely, the irregular contraction of the arteries or arterioles themselves, described in an earlier paper.

In types of artificial chambers, installed in rabbits' ears, which are invaded by new tissue, there are often arterioles that, for weeks, are unprovided with nerves and hence contract little, if at all. In many such chambers no arterial anastomoses survive. However, in this type of chamber, occasionally arterioles receive a nerve supply, and in such cases arterial anastomoses may survive. In every case in which such anastomoses have persisted in newly-formed tissue, there have been frequent reversals of flow in the connecting portion.

The effects of the ultra-violet radiations on Styela eggs. A. M. DALCO.

The M.D.L. installation for microphotography with U.V. rays (2537 Å) may be used for irradiating part of the Ascidian egg or certain of the various blastomeres up to the VIII-cell stage. The method was worked out with the aid of Dr. G. I. Lavin. The egg is placed in a drop of sea water near the edge of a thin quartz coverslip, which is itself put on the transverse arm of the mechanical stage. The coverslip is adjusted under the microscope in such a way that the part of the egg to be irradiated protrudes over the edge of the metallic stage arm which

acts as a protection screen for the rest of the egg. Attention should be paid to two sources of error: (1) the effect of hypertony due to evaporation of the drop and (2) to the reflexion of the rays by the objective lens of the microscope, which is easily eliminated by interposing some black paper during the irradiation. In exploring a considerable range of exposure no favorable effect of the irradiation could be found. If feeble, it produces a delayed disorganization of the embryonic layers. If stronger, it stops the cleavage with rapidity varying according to the dosage. In order to obtain stopping of the next cleavage, exposures of at least 10 minutes are necessary. When a division is suppressed, the cell may manifest a delayed attempt at cleavage, but this is always abortive. After exposure of the unsegmented eggs, deviations of the first cleavage plane may be observed. Observations of the movements of yolk and yellow pigment and the elongation of the cell-body indicate that the effect of the radiation is not primarily on the nuclear activity. That the influence of the rays is exerted on the surface protoplasm is shown by the transitory appearance of alterations of the surface film (small protuberances, "blisters") in coincidence with attempts at cleavage.

By means of this method, the division of one or more blastomeres of the II, IV, and VIII cell stages has been inhibited. The non-irradiated cells exhibit normal development with respect to mitotic rhythm and arrangement. Their capacity for differentiation, which seems rather poor when large blastomeres remain undivided in the germ, must still be studied in sections.

A correlation between gill surface and activity in marine fishes. I. E. GRAY.

The units of respiration in the gills of fishes are the numerous microscopic secondary lamellae which appear as thin, leaf-like plates set at right angles to the main axes of the primary lamellae. Within each plate lies a capillary network through which the interchange of gases takes place. Among fishes there are species differences, not only in the number of gills, but also in the number and length of the gill filaments (primary lamellae) and in the number of respiratory units (secondary lamellae). By determining the number of respiratory units per gram of body weight it is possible to obtain an estimate of the relative respiratory ability of different fishes.

There is a marked contrast in the number of respiratory units per gram of body weight between the active, surface, migratory fishes (mackerel, 2550; butterflyfish, 1725; menhaden, 1685) and the sluggish bottom fishes (flounder, 265; toadfish, 135; goosefish, 50). The number of respiratory units of fishes of medium activity fall between these two extremes (scup, 1325; sea trout, 1250; sea bass, 1110; eel, 900; sea robin, 800; puffer, 505; tautog, 440). A four hundred gram mackerel has a total of nearly three-fourths million respiratory units while a toadfish of the same weight has only fifty thousand. The number of respiratory units is also directly correlated with the amounts of sugar and hemoglobin in the blood.

*The distribution of lipid between the light and heavy halves of the *Arbacia* egg.* F. R. HUNTER AND A. K. PARPART.

Unfertilized *Arbacia* eggs were centrifuged for 10-20 minutes in an air turbine at approximately $10,000 \times G$ in a medium of graded density obtained by mixing sea water and 0.95 molal sucrose. The light and heavy halves which resulted were collected, packed in an air turbine, frozen, dried in a vacuum desiccator and weighed. This dried material was then extracted with ether, dried and again weighed. The loss in weight was taken as a measure of the amount of free fats and sterols. This material was then extracted with alcohol ether and again dried and weighed. This was considered to give a value for the bound lipid. In order to relate the amount of lipid to the number of halves, counts were made on suspensions of halves prior to drying. The following values expressed as mgs. of lipid per million halves were obtained: heavy halves—6.6 (ether fraction), 12.2 (alcohol-ether fraction); light halves—2.2 (ether fraction), 9.6 (alcohol-ether fraction). Thus, 75.0 per cent of the free fats and sterols, 56.0 of the bound lipids and 61.6 per cent of the total lipids are in the heavy halves. The sum of the total lipids in the two halves is equal to 30.6 mgs. per 10^6 cells which compares favorably with the value 34.1 mgs. per 10^6 cells calculated from the data given by Parpart (*Biol. Bull.*, 81: 296, 1941) for unfertilized, whole eggs. Similarly a comparison can be made between the sum of the bound lipids of the two halves and of the whole egg. Their values are 71.3 per cent and 77 per cent, respectively.

Evidence for enzymatic participation in the penetration of the human erythrocyte by glycerol. PAUL G. LEFEVRE.

Jacobs and his associates have reported that an amount of copper sufficient to cover only a very small fraction of the surface of the cells involved markedly inhibits hemolysis of human red cells in isotonic glycerol. This report concerns the extension of this finding to the effects of other substances which inhibit the same types of enzymes affected by traces of copper.

Following the pattern prescribed by Barron and Singer for identification of sulphydryl activity, iodine, mercuric ion, the arsenical Mapharsen, and p-chloromercuribenzoate were shown to inhibit hemolysis by glycerol, buffered at pH 7.1. This inhibition failed in the presence of cysteine, glutathione, or thioglycolic acid; and could be reversed by later addition of these substances at 2-3 times the concentration of the inhibitor, except with Mapharsen. These relations indicate strongly that active $-SH$ groups are involved in carrying glycerol into the cell. Though sensitive to the inhibitors mentioned, the hemolytic process was not affected by iodoacetate; this indicates that the sulphydryl groups involved are of the difficultly available type, not inactivated by the alkylating agents.

Since phosphorylation is apparently essential in transfer of sugars and other substances across the membranes of the kidney tubule and the intestinal cell, it is proposed tentatively that the enzymatic step involved in the present studies is the phosphorylation of glycerol. Adenosine triphosphatase, capable of this step, is present in the erythrocyte, and shows the same pattern of sensitivity to inhibitors as found in the present instance, as well as similar relations of activity to pH. Further, more decisive tests of the proposed identity of the enzymatic factor are planned.

"Accommodation" and opening excitation in nerve and muscle. PAUL G. LEFEVRE.

In his mathematical analysis of electrical excitation in 1936, Hill pointed out that the accommodative process (recession of threshold under the influence of a stimulus) itself accounted for the phenomenon of excitation at the anode at the "break" of a constant current. There seems to have been no attempt to test this neglected implication of Hill's theory: that "accommodation" is an essential prerequisite for "opening excitation" at the anode. This report concerns the occurrence of opening excitation in tissues showing no accommodation.

Following Solandt's practise, frog sciatic nerves were treated with citrate until they no longer showed any accommodation: their threshold was independent of the rate of increase of the excitatory current (delivered with Solandt's condenser-charge arrangement). In such preparations, in spite of the absence of accommodation, there was no difficulty in eliciting an anodal response at the cessation of a steady current. The same result was readily obtained with exposed sciatic nerves of anesthetized rats treated with citrate.

Frog sartorii, or the pharyngeal retractors of *Thyone*, if stimulated in the nerve-free regions, or following neural degeneration, also showed no accommodation. But all attempts to demonstrate any response at "break" in these muscles failed; this is in accordance with the predictions of Hill's theory. Only in the case of citrated nerves was seen the troublesome occurrence of opening excitation in the absence of any accommodation.

A further analysis of this matter is planned, to determine whether the results may be explained on the basis of a postulated fundamental difference between accommodation at the cathode and that at the anode; the latter persisting in the absence of the calcium ion required by the former.

A photometric study of the kinetics of fibrin formation. JOSEPH LEIN.

The clotting of fibrinogen solutions by thrombin was studied by measuring the optical density and light scatter as the process occurred. The results can be analyzed kinetically only when purified preparations were used. If other plasma proteins are present the degree of light scatter also depends on their concentrations. This is believed due to a trapping effect of non-clottable proteins by the fibrin as it is formed. The light scatter studies were particularly useful in the kinetic analysis of the clotting process.

The reaction was considered from a polymerization viewpoint, the fibrin representing the polymer formed through the action of thrombin on the monomer fibrinogen. First order reaction kinetics were employed. The following assumptions were made. (1) With constant thrombin concentrations the rate of increase of the polymer size is proportional to the fibrinogen concentration ($dN/dT = K_1 F$). (2) The rate of decrease of the fibrinogen is proportional to the fibrinogen concentration ($-dF/dT = K_2 F$). (3) The light scatter under the conditions of the experiment is proportional to the increase in particle size once it reaches the critical size (N_c) which first scatters light. ($LS = K(N - N_c)$). From these formulas a relationship was derived that included light scatter (LS), the initial concentration of fibrinogen (F_0), time (T), and time (T_0) for the polymer to reach a size (N_c) that would scatter light. The formula may be expressed as:

$$\log (K_1 K / K_2 F_0 e^{-K_2 T_0} - LS) = -0.434 K_2 T + \log K_1 K / K_2 F_0$$

The relationship was tested on a series of experiments in which the initial concentration of fibrinogen was altered, the thrombin being kept constant. The calculated values of the constants agreed with the experimental values within a 6 per cent average deviation. It thus appears that the course of the reaction may be considered to be molecular and that thrombin acts as a true catalyst, not forming part of the final fibrin product.

The effect of iodacetate on the changes in muscular latency induced by activity.

A. SANDOW. No abstract submitted.

Formation of the nuclear membrane and other mitotic events in Chaos chaos Linn and Chaos neos (new species). A. A. SCHAEFFER.

The mitotic stages of the amebas mentioned are easily followed in the living animal. The principal stages are the following: 1. the nucleus about to divide swells up to about 6 times its former volume. 2. The chromatin grains (300 to 600 in number) gradually disappear, as if going into solution. Some of these grains coalesce before going into solution. 3. A new mass of small grains (about 2500) appear, before all the larger grains of the so-called resting nucleus have disappeared. These small grains arrange themselves first as a lens-shaped cloud, then as a plate of about 2 grains thickness. At this stage the plate of grains is occasionally seen to be indistinctly divided into at least 4, possibly as many as 8 or 12, smaller groups of equal size. 4. This plate of grains then separates into 2 plates which rapidly move apart. 5. Fibers analogous to, if not identical with, spindle fibers, appear between the plates, as the plates separate. Fibers also appear on the other face of the plates. All fibers are at first horizontal and parallel. 6. The plates separate and the inter-plate fibers lengthen until the plates are separated to about 2 or 3 times their diameter when, because of the streaming of the protoplasm, the plates are torn apart. During this time the nuclear membrane breaks into pieces which eventually completely disappear. 7. The separated plates, still granular, become bent like a concavo-convex lens, with polar fibers still attached. The granules soon disappear, leaving a very thin, perfectly homogeneous flat disk that shows a brilliant blue green color when seen on edge. No refractory edge can be made out. This stage is difficult to see. 8. After a few minutes the disk shrinks in diameter and is thrown into rope-like folds around the periphery. 9. Very soon thereafter a refractory edge begins to appear as the folds disappear. 10. Very fine grains presently begin to appear until about 1,400 are formed. Many of these coalesce to form larger grains until only about 600 to 700 remain in the newly formed daughter nucleus. (Further reduction in number may occur during the next few hours.) While the small grains are appearing, the edge of the nucleus becomes more and more refractory until in the new nucleus it is seen as the new nuclear membrane. The steps outlined here require about 28 minutes.

The mitotic events of *Chaos diffuens*, as far as they have been observed, are practically identical with those of the above-mentioned species, except for size.

Correlated histories of individual sense organs and their nerves, as seen in living frog tadpoles. CARL CASKEY SPEIDEL.

In the living frog tadpole it is possible to make daily observations on the same individual nerves and sense organs of the lateral-line for many weeks or months. By suitable operations

some sense organs may be deprived of their nerve supply (nerveless organs), and conversely, some aberrant lateral-line branches may be induced to grow without reaching any sense organ (organless nerves).

Prolonged observations of nerveless organs (1 to 21 months) reveal the following: (1) During the first two months in regenerating or growing zones the sense organs are largely independent of their nerve supply. They grow and divide readily. (2) During later months, however, regressive changes of atrophy and degeneration take place. The organs become smaller. Some degenerate and disappear. Occasionally, however, a nerveless organ may persist for more than a year.

Prolonged observations of organless nerves reveal the following: (1) During the first two months they are largely independent of the sense organs. They grow and become provided with both neurilemma and myelin sheaths. (2) During later months, however, regressive changes ensue. The myelin sheath is not maintained on any functionless fiber. It becomes thinner and ultimately disappears. The unmyelinated fiber resulting may then itself degenerate, leaving only a collapsed neurilemma tube.

Thus, the structural integrity of both lateral-line sense organ and nerve fiber is definitely correlated with the successful establishment of a functional relationship between the two.

Sense hairs and orange granules are specialized structures of lateral-line organs. The behavior of both of these under various experimental conditions indicates their relative independence of nerve influence.

Many other histories involving nerve and sense organ relations in wound zones have also been recorded. Illustrative cine-photomicrographs have been made.

The effect of prolonged starvation on the lipids in Phascolosoma gouldii. CHARLES G. WILBER.

It is known that the muscle in vertebrates serves as a storehouse of fats and that during starvation the fats in muscle decrease whereas the fat in various internal organs is not changed. Whether this is true for invertebrates is not known.

In order to throw light on the problem, worms (*Phascolosoma gouldii*) were starved for one month and then the whole worm, the muscle, and the perivisceral fluid respectively were analyzed for phospholipid, for cholesterol, and for fatty acid. These results were compared with the results of similar analyses made on control worms.

It was found that in the whole worm there was a loss of all lipid constituents. In the perivisceral fluid, phospholipid and fatty acid decreased greatly, but cholesterol did not decrease. In the muscle there was an apparent increase in lipid material which can be explained on the basis of absorption of some of the tissue. In muscle the fatty acid is decreased, as is clear from the larger lipocytic coefficient of the muscle of starved worms.

It is concluded that the perivisceral fluid serves as a storehouse of lipid in *Phascolosoma* and that the muscle does not. Moreover, it seems that phospholipid and fatty acid are used during starvation. Whether cholesterol is also used is not certain. *Phascolosoma* differs, therefore, from the vertebrates in the use of phospholipid during starvation and in the fact that muscle is not the important storehouse of fat.

Protoplasmic clotting in isolated muscle fibers. ARTHUR A. WOODWARD.

Isolated muscle fibers provide a material favorably adapted to the quantitative study of protoplasmic clotting. The cut ends form clots which pass in waves over the length of the fiber. The rate of the clotting reaction can be measured and is expressed as mm. of fiber converted into clot per minute. Single fibers are teased from the adductor magnus of *Rana pipiens*; all solutions used are kept at pH 7.1–7.4 with glycine buffer.

In Ringer's solution, used as a standard for comparison, the rate of clot formation is constant for a given fiber and varies only moderately from fiber to fiber within a muscle. The normal rate is about 0.050 mm./min.

Ca ion causes a very rapid clot formation; in this case it is shown that the rate is largely a function of the speed with which Ca diffuses into the end of the fiber, the protoplasm clotting with great rapidity once it is exposed to free Ca ion.

The clotting process is relatively insensitive to pH changes in the region from pH 5 to pH 9; above and below this the rate increases very rapidly. Near the regions in which thrombin is inactivated liquefaction has been observed under certain conditions.

Solutions of crystalline trypsin cause clot formation at a rate averaging about 50 times that of the control. In the absence of Ca, trypsin produces only a slight increase over the control. Crystalline chymotrypsin is much less active than trypsin and also has very little effect in the absence of Ca. Preparations of crude papain cause clot formation at a moderately high rate; addition of glutathione increases the effect to the magnitude of that produced by trypsin. Absence of Ca has no effect on the action of papain.

AUGUST 24

Some aspects of the histology and physiology of luminescence in "railroad worms."

JOHN B. BUCK.

In the Uruguayan "railroad worm," *Phrixothrix*, the lateral photogenic organs are small compact ovoid masses of small dense cells near the posterior edges of the segments somewhat above the spiracular level. The organ is apparently supplied by one trachea ramifying profusely between the cells. There are no end-cells. Large oenocyte-like cells are present near some of the lateral photogenic organs and elsewhere.

In *Phengodes*, a close American relative of *Phrixothrix*, the lateral organs are in the posterior ends of horizontal rolls of tissue which extend along the segments ventral to the spiracles. Light is also emitted along the dorsal posterior edges of most of the segments. Both lateral and dorsal organs apparently consist of loose aggregations of very large oenocyte-like cells without end-cells or special tracheal supply. Similar cells are present in small numbers in parts of the body not regarded as luminous but not in the lateral tissue roll except in the region which emits light. Further evidence is furnished by the observation that the light of *Phengodes* can be seen microscopically to come from clusters of round or oval spots corresponding in shape, position, number, and size to the oenocyte-like cells.

The photogenic organ of *Phrixothrix* is very similar to that in the larval firefly and agrees with the generalization that luminous beetles which produce a lingering glow rather than a short flash, have organs of relatively simple structure without end-cells.

The photogenic organs of *Phengodes* are the simplest yet known in insects and represent the first time that bioluminescence has been ascribed to oenocytes. A corresponding physiological simplicity may be the fact that the light is continuous.

Phengodes dims in the vapor of 10^{-2} and 10^{-3} M. HCN at about the same rate as luminous bacteria, and faster than fireflies.

Effect of caffeine concentration upon retardation of Arbacia development. RALPH HOLT CHENEY.

Sea urchin eggs and sperm were subjected to eight different concentrations of caffeine-in-sea-water for 15 minutes, then mixed for fertilization and the developmental rates in S.W. and S.W.C. were compared with the normal rate of untreated ova and sperm. Observations were made at intervals during a three-day period. Normal time rates were accepted as stated by E.B. Harvey, 1940 (*Biol. Bull.*, 79, (1) Plate II, photographs 16-32 inclusive).

All eggs utilized in a single experiment were obtained from the same female and all sperm from one male. Eggs and sperm were shed directly into S.W. or S.W.C. prior to mixing. The series of six combinations presented in 1942 (*Biol. Bull.*, 83) were repeated and extended to a full three day period as follows:— $N\text{♀} \times N\text{♂}$, $N\text{♀} \times C\text{♂}$, $C\text{♀} \times N\text{♂}$, $C\text{♀} \times C\text{♂}$, all developed in normal sea water after the original immersion of fifteen minutes after shedding as indicated into S.W. or S.W.C. In the cases of $C\text{♀} \times N\text{♂}$ and $C\text{♀} \times C\text{♂}$, each was developed also in S.W.C.

Results indicated that the period of immersion (15 min.) in the caffeine concentrations employed prior to mixing the gametes did not render the ova non-fertilizable subsequently nor destroy the ability of the sperm to fertilize. Eggs and/or sperm, however, were not unaffected at least by higher concentrations, since $C\text{♀} \times C\text{♂}$ cultures, although they did form the fertilization membrane when mixed and developed in uncaffeinated S.W., the fertilized ova never survived longer than the early cleavage stages.

Plutei developed in normal time and form in all of the six combinations of 0.002 per cent and 0.004 per cent S.W.C. Gametes shed into two per centum S.W.C., in each of the four combinations developed in S.W. formed the F.M. but showed retarded development and in no case reached the pluteus stage before death. In the two combinations developed in S.W.C., the F.M. was not formed. Intermediate percentages used between these extremes of concentration gave intermediate effects indicating that in general, the effects were directly proportional to the concentration of the caffeine.

Other experimental series subjected normally fertilized ova ($N\varnothing \times N\sigma$ in S.W.) which had developed to a desired stage of development, to the different concentrations of caffeine-in-S.W. Results here indicated similarly that the retardation effect upon the development time was proportional to the concentration.

Shape changes in the denuded Nereis egg preceding first cleavage. ALBERTA T. JONES.

It is known from Hoadley (1934) that *Nereis* eggs undergo a series of amoeboid changes prior to first cleavage. Neither the reason for this phenomenon nor the exact pattern followed has been completely described. It is the purpose of this paper to report the principal findings on the pattern of shape changes up to first cleavage in the egg of *Nereis limbata*. The denuded egg was used to eliminate the complications of membrane and external jelly.

Gametes were taken from animals caught the previous night in Eel Pond (Woods Hole) and artificial insemination was carried out. The fertilized eggs were denuded by treatment with alkaline 0.53 molar NaCl solution brought to pH 10.5 by addition of Na_2CO_3 . This is the method used by Costello (1939 and 1945). Observations began when the denuded eggs were rinsed free of alkali and the first polar body had formed. Outline drawings of the eggs were made with a camera lucida at three minute intervals. To serve as reference points, the position of polar bodies and oil droplets was indicated.

Hoadley states:—"pulsations of the (*Nereis*) egg are of two sorts, one of which is quite extensive and results in general distortion of the sphere, and the other of which results in surface irregularities which appear more or less localized." The shape changes discernible in denuded eggs seem to correspond to Hoadley's first category. A consistent pattern of sequences, different from those described by Hoadley for the intact egg, has been found. The sequences include such general distortions as: (1) polar flattening followed by rerounding; (2) elongation in the polar axis followed by rerounding; and, (3) elongation in the equatorial axis followed by formation of the first cleavage plane. The magnitude of these changes, in comparison with the pulsations observed by Hoadley, may be attributed to the absence of jelly mass and membrane.

It may be concluded that shape changes in the denuded *Nereis* egg prior to first cleavage proceed (1) according to a definite pattern; and (2) always with a particular relation to the polar axis of the egg.

Hormone control of dehydrogenase activity of Crustacean tissues. ELOISE KUNTZ.

Sea water extracts of the sinus glands of *Libinia emarginata*, *Homarus americanus*, *Uca pugilator* and *U. pugnax* and similarly prepared extracts of the central nervous system of *Libinia*, *Homarus* and the arachnid, *Limulus polyphemus*, were made. These were boiled and centrifuged and the supernatant fluid was used. The extracts were tested for their effect upon dehydrogenase activity of gastric gland and muscle of *Libinia*, *Homarus* and *Limulus*, which were measured in Thunberg tubes with methylene blue as the hydrogen acceptor.

The effect of sinus gland extract was dependent upon the concentration. Half of a *Libinia* sinus gland doubled the rate of methylene blue reduction, but the reduction rate rapidly fell to slightly above that of the controls with increasing concentrations. Half of a *Uca pugilator* sinus gland also doubled the reduction rate, but activity remained high for concentrations of 2 sinus glands, falling to the level of the controls at 6 sinus glands. Here it remained. *Uca pugnax* showed strong inhibitory action in concentrations of 6 to 12 sinus glands. The character of the curves suggests the possibility of two active substances which vary in relative proportions in different species.

Central nervous system extracts of *Homarus*, *Libinia* and *Limulus* in concentrations of 0.7 mg. tissue per cc. strongly stimulated dehydrogenase activity. Extracts of other tissues were ineffective at several times this concentration.

Localization of hormone production within the nervous system was demonstrated. In *Homarus* the circumoesophageal ganglia and second ventral ganglion were most effective. The brain and suboesophageal ganglion were ineffective. The remainder of the ventral cord had a relatively weak action. All parts of the circumoesophageal ring of *Limulus* were effective.

An antagonistic action of sinus gland and nervous system extract was demonstrated. The addition of sinus gland hormone to a system stimulated by central nervous system extracts depressed dehydrogenase activity to that of the controls.

A comparative study of cholinesterase activity in normal and genetically deficient strains of Drosophila melanogaster. DR. F. POULSON AND E. J. BOELL.

Cholinesterase activity has been determined in late embryos of several strains of *Drosophila melanogaster* by means of the cartesian diver technique which measures the evolution of CO_2 from Ringer-bicarbonate solution following hydrolysis of acetylcholine in the presence of an atmosphere of 95 per cent N_2 and 5 per cent CO_2 . Timed eggs from a stock of the deficiency known as Notch⁸ crossed to Canton-S wild strain ($\text{N}^8/+$) were dechorionated by Slifer's hypochlorite method and classified as normal or Notch-deficient. At 24 hours normals are larvae ready to hatch, while the deficient male embryos are strikingly abnormal and possess a nervous system about three times normal size. The ratio of types is 3 normal: 1 abnormal. Embryos were cut up and placed in divers containing 1 mm.³ Ringer-bicarbonate and 0.5 mm.³ of 1.5 per cent acetylcholine. Although it is possible to carry out measurements on single embryos, two to five embryos per diver were used in most experiments. Readings were made at ten minute intervals for one hour after the divers had reached thermal equilibrium.

A series of four determinations on 24-hour normals gave an average of 12.8 m. μ l. CO_2 /embryo/hour. A series of five determinations on Notch⁸ deficient male embryos gave an average of 34.0 m. μ l. CO_2 /embryo/hour. The cholinesterase activity of Notch embryos is 2.7 times that of normal, which is nearly the same as the volume ratio of Notch/normal nervous systems, 3.3 as determined by planimeter from camera lucida outlines of sections. Thus cholinesterase activity is proportional to volume of nervous tissue. Notch-deficient embryos of other strains have given similar results. Thus the Notch male nervous system while abnormal in size and morphology is biochemically normal with respect to cholinesterase. A first step has been made in studying the rate of increase with development of cholinesterase activity in both normal and deficient embryos. At 18.5 hours the activity of the Notch embryo is 8.3 m. μ l. CO_2 /hour, that of normal 3.8 m. μ l. CO_2 /hour. In the unhatched Notch embryo at 48 hours the activity increases to 51.0 m. μ l. CO_2 /hour.

As checks, cholinesterase activity of unfertilized eggs and methyl butyrase activity of normal and Notch embryos were measured and found to be negligible.

To determine the location of cholinesterase in normal embryos, central nervous systems were dissected out and their cholinesterase activity measured separately from the remaining portion of the embryos. One determination has given a value of 17.0 m. l./N.S./hour. The value for the remnant is 2.6 m. μ l./hour. Since the central nervous system makes up not more than one-sixth the embryonic volume the cholinesterase activity there is roughly forty times that in the remnant.

*Possible metabolic and physical chemical factors in the production of the injury potential in spider crab nerve.** A. M. SHANES.

In contrast to frog sciatic nerve, spider crab nerve is permeable to both potassium and chloride ions and to a lesser extent to sodium. The relationship between metabolism and the potentials therefore cannot be the same as in frog nerve which is highly impermeable to chloride and other small anions as well as to sodium. This is confirmed by the following observations: (1) Although 0.002 to 0.0006 M iodoacetate (IAA) produces a continuous slow fall in

* Aided by grants from the Penrose Fund of the American Philosophical Society and from the American Academy of Arts and Sciences.

potential in oxygen, 0.02 M pyruvate is unable to counteract this inhibition; (2) pyruvate accentuates IAA inhibition of the post-anoxic recovery of potential; (3) the decline of potential in nitrogen is more rapid than in frog nerve and is not hastened by IAA; (4) glucose does not retard the fall in potential during anoxia and inhibits a recovery in oxygen. The inhibitory effects of pyruvate and glucose may be the result of acid production, for 5 per cent CO_2 lowers the potential in this system in contrast to its effect in frog nerve.

These results provide a basis for understanding some peculiarities of carbohydrate metabolism in spider crabs. For example, blood sugar levels average only 1 mg. per cent, nerve glycogen ranges from 500 to 2,000 mg. per cent wet weight (Kleinholtz, unpublished), and the breakdown of glycogen is reported to be largely to simple sugars as well as to lactic acid.

The potassium content of these fibers is known to be high and the potential is inversely related to the extracellular potassium concentration. Consequently the injury potential is probably a potassium concentration potential as in frog nerve. In yeast (Rothstein and Haeger, 1943) potassium retention is stoichiometrically related to hydrogen ions lost to the medium when glucose is assimilated to form reserve carbohydrate. This mechanism may explain the high levels of both glycogen and potassium, and hence the metabolism-potential relationship, in spider crab nerve.

Some effects of tannic acid on osmotic hemolysis. T. H. WILSON AND M. H. JACOBS.

Human erythrocytes are less easily hemolyzed in hypotonic solutions of NaCl in the presence of tannic acid than in its absence. The salt solution employed in the present experiments ranged from 0.091 to 0.069 M and those of tannic acid from 1/800 to 1/51,200 per cent. Even at the lowest of these concentrations of tannic acid there was a marked protective effect. In hypotonic solutions of Na_2SO_4 , ranging from 0.045 to 0.031 M and with the same concentrations of tannic acid as before, the effect was the exact opposite, hemolysis invariably being increased except at the lowest concentrations of tannic acid. Very similar effects, somewhat complicated by the permeability of the erythrocyte to ammonium salts, were obtained with NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$, respectively.

In the light of results obtained with molecular films of proteins by Schulman and others, the action of the tannic acid in the chloride solutions might be explained either by a strengthening of the cell surface or by a decrease in its permeability to hemoglobin. Such an action in the case of the sulfate solutions is not necessarily excluded, but it seems to be overshadowed by another effect of a different nature, namely, the decreased permeability to anions produced by tannic acid, described elsewhere by Jacobs, Stewart and Butler. Since swelling of the erythrocyte is known to be opposed by the exchange of bivalent sulfate ions from the outside for univalent anions from the inside, tannic acid, by hindering this exchange, might in this particular case indirectly favor hemolysis, despite its more direct protective effect on the cell surface.

The effect of roentgen radiation on protoplasmic viscosity changes during mitosis. WALTER L. WILSON.

Roentgen radiation has a marked effect on the protoplasmic viscosity of the dividing sea-urchin egg. If the eggs, or sperm, or both the eggs and sperm of *Arbacia punctulata* are irradiated before fertilization, then the normal pattern of viscosity change is altered. In the control the viscosity was low shortly after fertilization, then increased to a peak at 15 minutes (23° C.). It remained high for 6-10 minutes and then decreased. This decrease was markedly retarded by irradiation of the sperm or eggs (11,300 r at 6400 r/m), or both the sperm and eggs (5,000 r at 6400 r/m) before fertilization. In these experiments the viscosity remained high two or three times longer than in the controls. In three experiments out of ten in which irradiated eggs were fertilized with normal sperm, the viscosity increased to a value almost twice that of the controls.

Biological specificity and the synthesis of native proteins. D. WRINCH. No abstract submitted.

PAPERS READ BY TITLE

The effects of massive doses of ergonovine maleate upon the smaller blood vessels as seen directly with the microscope in the living mammal.*¹ RICHARD G. ABELL.

In these experiments, as in those described above, the blood vessels were studied in transparent 'moat' chambers in rabbits' ears. All injections were of 30 mgm., and all were made intravenously. Three mgm. in a rabbit corresponds to 60 mgm. in a man, which is 600 times the clinical intravenous dose. In these experiments the injection of 3.0 mgm. caused complete arteriolar constriction for 3 to 4 minutes. The larger arterioles (80 to 90 microns in diameter) remained narrowed to approximately one-half of their control diameters for 3 to 4 hours. A temporary constriction of the venules to from 0.5 to 0.8 of their control diameters occurred. In addition, this amount of ergonovine caused thickening of leukocytes to the walls of the arterioles, capillaries and venules. The degree of sticking varied widely in different rabbits; in some cases it was slight; in others large numbers of leukocytes stuck to the walls of the capillaries and venules, and emigrated into the surrounding tissue. In one rabbit injections of 30 mgm. were followed by the formation of leukocytic emboli, which blocked many of the capillaries and venules and formed thrombi. The reaction was reversible and the thrombi usually disappeared within approximately 4 hours following the injections. This is in accord with the flow toxicity of ergonovine, and its failure to produce gangrene on repeated injections.

As shown by the work of numerous investigators, two other ergot alkaloids, ergotamine and ergotamine, do produce gangrene on repeated injection. Such gangrene also occurs in ergot poisoning and is due to obliterative endarteritis and thrombosis. The formation of these thrombi is usually attributed to prolonged constriction of the small arteries, and interruption of the blood flow, but this is entirely hypothetical.

In the present experiments thrombi were formed due to the increase in stickiness of the endothelium toward leukocytes and of the leukocytes toward each other.

Perhaps gangrene produced by ergot and its more toxic alkaloids may be caused by a more severe and prolonged reaction of the type described above.

Secretory cells in the branchial epithelium of fishes. GERRIT BEVELANDER.

It was shown by Smith (1930, 1931, 1932) that the osmotic regulation of the body fluids in fresh and salt water teleosts and in elasmobranchs is effected considerably by the extrarenal excretion of salt (NaCl and KCl) under conditions that probably involve considerable osmotic work. It was further inferred that this exchange occurred in the gills. A previous study of the branchial epithelium in an extensive and widely divergent group of fishes (Bevelander (1935), led this writer to conclude that the only specialization occurring in the branchial epithelium of fishes consists in a thicker epithelium in the elasmobranchs than in teleosts and the presence of numerous mucous cells in all species examined. The cells which we described as mucous, were alleged to be "chloride secreting" cells in *Anquilla* and in some fresh water teleosts, but not in elasmobranchs by Keyes and Willmer (1932).

A re-examination of this problem included the experimental stimulation of secretion of the cells in dispute in representative teleosts and elasmobranchs. These cells were then subjected to a number of histochemical tests and were shown to be positive for mucin. Further, the oral and opercular membranes were also examined and it appears unlikely on the basis of structure that they are concerned with osmotic regulation.

In order to comply with the observed physiological data, the cells which are responsible for extrarenal excretion must be in intimate relation with the blood supply and the external milieu, they must be very extensive to account for the considerable work performed, and finally they must be present in teleosts and elasmobranchs. Our observations reaffirm the absence of any specialized structures; the only cells which comply with the three criteria required are the

* 'Ergotrate' (Ergonovine Maleate, U.S.P., Lilly).

¹ This work was aided by a grant made by Eli Lilly and Company to the Department of Anatomy of the University of Pennsylvania Medical School.

respiratory epithelial themselves. It is further suggested that the observed conservation of urea in the elasmobranch gills may be affected by the relatively thick respiratory epithelium which covers the gill filaments.

A modified Crompton formula for the latent heat of vaporization. ALBERT P. MATHEWS.

The general formula for the latent heat, L , which I have found is

$$(1) \quad L = CR'T \ln(d/D)$$

R' is the actual value of the gas constant in the liquid phase, constantly falling as molecular co-aggregation increases with falling temperature. d is the liquid density and D that of the vapor, C is a constant peculiar to the substance, often not far from 2.

The values of C and R' are obtained from the following general formulas which apply to all non-associating substances with the possible exceptions of hydrogen and helium.

$$(2) \quad C = C'(R/R_e)(L/(L-E))_e$$

$$(3) \quad C' = 1.1292 - (3/8)S + (9/64)S^2 = 0.8792 + ((3/8)S - 0.5)^2$$

S in (3) is the critical coefficient: RT_c/p_cV_c in which R has its ideal value. S may be computed by (4):

$$(4) \quad S = [(d_m RT_c / M p_c) + 16((T_c - T)/T_c) - 12((T_c - T)/T_c)^2] / [1 + 5.158((T_c - T)/T_c) - 3.158((T_c - T)/T_c)^2]$$

d_m is the mean density of saturated vapor and liquid at temperature, T ; p_c and T_c the critical pressure and temperature.

$$(5) \quad (L/(L-E))_e = 1 + 64R^2/27S^2R_e^2$$

$L-E$ is the internal latent heat of vaporization. (5) is obtained from (6).

$$(6) \quad ((T/p)(dp/dT))_e = (L/E)_e = 1 + (27S^2 R_e^2)/64 R^2$$

$$(7) \quad R'_e/R = (512 - 64S + 216S^2 - 27S^3)/512S$$

R'_e in (1) is obtained from (8).

$$(8) \quad (L/(L-E))_e = R'_e/R_e = 1 + ((L/(L-E))_e - 1)((9/16)(T/T_c) + 7/16)(T/T_c)^2$$

$$(9) \quad R'_e = R'_e/(L/(L-E))_e$$

At absolute zero $L/(L-E)$ is 1 and it advances with temperature as co-aggregation diminishes. In the ideal state C , S and $(L/E)_e$ are 1. When S has its highest value of 4 in a normal substance $(L/E)_e$ will be 7.3346 and, when S has its lowest value of 3/8, $(L/E)_e$ will be 4. S may be calculated also from the latent heat of vaporization at any temperature, C' being equal to $(L-E)/RT \ln(d/D)$ by (10):

$$(10) \quad S = (8/3)(0.5 + \sqrt{(C' - 0.8792)})$$

Obtained from (3) above; E being taken, with small error usually, as equal to $p(V-v)$.

Formula (1) above is an easier and more accurate way of computing the latent heat of vaporization than by the thermodynamic equation: $L = (Td p/dT)(V-v)$. The results by (1) agree usually within 1 per cent with the experimental determinations at the normal boiling point as made by J. H. Mathews and others. The derivation of all the foregoing formulas will be given in the full papers together with examples of application to specific cases and also the general formula for the Cailletet and Mathias law (11):

$$(11) \quad d_m = d_e[1 + (5.158 - 16/S)((T_c - T)/T_c) + (12/S - 3.158)((T_c - T)/T_c)^2]$$

Heat Death. PAUL R. ORR.

I. Time-temperature relationships in marine animals.

Temperature as an intrinsic ecological factor which determines, to a great extent, the abundance, life cycle, and distribution of marine organisms, is a well-established fact. However, the duration of exposure required to produce death at each temperature in the effective series has not been taken into consideration. Thus, in order to state accurately the conditions of heat death, it is necessary to plot a curve in which both variables, temperature and time, are represented.

Heat death curves have been plotted for *Uca puqilator*, *Asterias forbesi*, *Ophioderma brevispinum*, *Arbacia punctulata*, *Nassa obsoleta*, *Fundulus heteroclitus*.

All of the curves have approximately the same shape. For a relatively slight rise in temperature there is a marked drop in the length of exposure necessary to cause death. This relationship is not one of direct proportionality.

II. Differential response of the entire animal (*Rana pipiens*) and several of its organ systems.

Whether we are dealing with cells or multicellular organs and tissues, or the organism as a whole, we are confronted with the fact that not all of the cells, organs, etc., have the same sensitivity to heat. An animal exposed to excessive heat for a length of time to cause complete loss of excitability might well be pronounced dead, for it never again will show any signs of life as a complete organism. Yet there are parts of the complex animal that are "alive."

The animal as a whole, the tadpole, sciatic nerve, sartorius and gastrocnemius muscles, and heart were separately studied, and curves were plotted for the heat death points of each. The data show that in the adult animal the order of death is: (1) the organism as a whole; (2) the muscular system; (3) heart, and (4) nervous tissue.

All heat death curves plotted are of the same shape, showing a sudden drop followed by a gradual approach to a constant level.

III. The effect of high temperatures on heart rate in *Venus mercenaria*.

In the clam heart (*Venus mercenaria*) we have an automatic mechanism by which the effect of heat can be studied. By subjecting excised hearts to a series of high temperatures and noting the heart rate it was possible to determine the lethal point for each temperature and thus plot a curve showing time/temperature relationship.

For the clam heart the same general type of curve was found as shown in previous studies on marine animals and frogs. That is, there is a point at which the hearts will beat for a relatively long period of time; then as they are subjected to higher temperatures there is a rapid decrease in heart rate, followed by a leveling off to a constant rate.

Penetration glands in tapeworm Onchosphaeres. W. MALCOLM REID.

Although various types of cystogenous and penetration glands have long been figured and studied as a part of the internal structure of trematode cercariae and miracidia, they have not been recognized in the onchosphere stage of cestodes. A pair of such glands has been found in the fowl cestodes *Raillietina cesticillus* (Molin), and *Choanotaenia infundibulum* (Bloch) and in a herring gull cestode *Hymenolepis* sp. Although these glands may be seen under favorable conditions without special stains, they respond in the same manner to vital stains as do trematode glands, showing up best with Nile blue sulfate and neutral red. The gland stretches to the posterior end of the larva, where it appears to be anchored. The secretion pores are located near the anterior and slightly to the side and above the middle pair of hooks when these hooks are oriented with the points directed anteriorly and downward. The granular contents may be seen to move about as the general contour of the glands is changed by the violent contractions associated with hook movements and at times some of the secretion may be seen exuding from the pores. A single nucleus is located near the middle of each gland, and the two glands are connected by a narrow isthmus near the posterior end.

The nature of the secretion has not been determined but it is possible that it assists the larva in penetration since this granular substance is given off at a time in the life cycle when the six-hooked embryo must break out of the covering membranes of the egg and penetrate the gut of an arthropod intermediate host.

Intensity-duration relation in stimulation by light. F. J. M. SICHEL AND P. B. ARMSTRONG.

The excised sphincter pupillae of many vertebrates will respond by constriction to stimulation by visible light of suitable wave-lengths. In these experiments the *sphincter pupilla* of the eel, *Anguilla rostrata*, was used. The sphincter was excised from small adults, 15 to 18 inches in length.

The sphincter was pinned out, anterior surface uppermost, on white beeswax. It was illuminated for observation by transmitted red light, to which the preparation is insensitive. The source of light for stimulation was a tungsten filament lamp maintained at constant voltage. This was focussed on the preparation obliquely from above. The intensity of the stimulating light was varied by Wratten neutral filters and a neutral wedge. The duration of the stimulating flash was controlled by a shutter manually operated and timed by a stop watch. The criterion of threshold was the smallest contraction visible through a low-power microscope. An eyepiece filar micrometer was used to advantage in determining the threshold stimulus. The preparation was bathed in a Ringert's fluid and permitted to become dark-adapted before each experiment.

The threshold was found to be a function of the duration and of the intensity of the stimulating flash. The intensity-duration relation conforms with Hill's theory of excitation for rectangular stimuli. The chronaxies averaged about 12 seconds, the range being from about 6 seconds to 20 seconds. In terms of the reciprocity law this would mean that the law holds reasonably well for flashes shorter than, say, 10 seconds. At longer durations the deviation is, in direction and amount, what would be expected on the basis of Hill's equation for excitation. There is a definite rheobase, or minimal intensity of the stimulating flash below which excitation is never produced, even for very long exposure times.

The pattern of the intrinsic palmar musculature. WILLIAM L. STRAUS, JR.

The intrinsic palmar musculature of tetrapod vertebrates comprises two fundamental series: (1) a superficial, arising from *fascia or tendon*, and showing variable tendency toward stratification, and (2) a deep, arising from *bone* and always arranged in two layers separated by the deep palmar nerves and vessels. Between the two series lies the mid-palmar space.

In urodeles (*Necturus maculosus*, *Cryptobranchus alleghaniensis*), the superficial series is a single layer (*flexores breves superficiales*) arising from the dorsum of the long flexor tendon. The deep series is composed of a superficial (*contrahentes* or *adductores*) and a deep (*flexores breves profundi*, *intermetacarpales*, *interphalangeus III?*; in *Cryptobranchus* also *flexores breves minimi*) layer.

In lizards (*Sceloporus spinosus*, *Ctenosaura similis*), the superficial series tends to form two layers—a superficial (*flexores breves superficiales*, *marginal abductors*), arising from the transverse carpal ligament, and a deep (*lumbricales*), arising from the long flexor tendon; in *Sceloporus*, however, such lamination is incomplete, for fibers of the superficial layer also arise from the long flexor tendon. The deep series again exhibits superficial (*contrahentes*) and deep (*flexores breves profundi*) layers.

In mammals (*Didelphis virginiana*, *Macaca mulatta*, *Homo*), the superficial series forms two distinct layers—a superficial (*abductor pollicis brevis*, *flexor pollicis brevis*, *opponens pollicis?*, *palmaris brevis*, *flexor V brevis*, *abductor V*; in *Didelphis* also a *flexor brevis manus*), largely from palmar aponeurosis and transverse carpal ligament, and a deep (*lumbricales*), from the deep long flexor tendon—separated by the superficial palmar vessels and nerves. The deep series again has superficial (*contrahentes*; only *abductor pollicis* in man) and deep (*interossei*, *opponens V*) layers.

Muscular homologies, at least between vertebrate classes, cannot be reasonably extended beyond comparison of entire palmar layers. Direct homology of individual muscle units is profitless and probably invalid.

The toxicity of a mixture of high molecular alkyl-dimethyl-benzyl ammonium chlorides to Fundulus. CHARLES H. TAFT.

The mixtures of high molecular alkyl-dimethyl-benzyl ammonium chlorides used is sold by the Winthrop Chemical Company under the trade name Zephiran Chloride* for use as an anti-septic or disinfectant.

Taft and Strandtmann (1945. *Fed. Proc.*, 4: 136) showed that under laboratory conditions this material is an efficient larvicide for the mosquito *Culex quinquefasciatus* and *Aedes aegypti* in dilutions up to 1:250,000. It seems desirable to determine its toxicity to some of the animals it might be brought in contact with if used for this purpose. Taft (1946. *Texas Rpts. on Biol. and Med.*, 4: 25) has reported its toxicity for various invertebrates.

To determine the toxicity by injection fundulus were injected intraperitoneally with different doses of one per cent solution; 0.25 cc. killed 17 out of 22, 0.05 cc. killed 15 out of 17 while 0.1 cc. killed 24 out of 24 fundulus. When these fish died they were darker than the controls and in many of them the abdomen was red about the site of injection. When the abdomen was opened there was frequently a greenish fluid present and the viscera had the appearance of having been cooked. The liver, gall bladder, heart, kidneys, and gills appeared normal.

Other fundulus were placed in finger bowls containing 225 cc. aerated sea water with different concentrations of the drug. When the fish were placed in dilutions of from approximately 1:2,500 to 1:100,000 all the fish died in from 35 to 105 minutes. On autopsy there were no significant gross changes. A dilution of 1:225,000 killed 25 per cent of the fish while 1:500,000 did not kill any of the fish exposed to it.

To determine the effects of longer exposure to the drug several fundulus were placed in battery jars in aerated sea water solution of from 1:100,000 to 1:400,000 and observed at the end of 24 hours. All the fish exposed to 1:100,000 and 1:200,000 were found dead. Twenty-five per cent of those exposed to 1:300,000 died while the 1:400,000 solution failed to kill any fish. It is evident that the effective range of this drug when employed as mosquito larvicide might be deleterious to fundulus.

Further evidence of polypoidy in the conjugation of green and colorless Paramecium bursaria. RALPH WICHTERMAN.

In a study of the time-relations of the nuclear events in living and Feulgen-stained preparations through conjugation, instances of polypoidy were encountered. Polypoidy was first recorded in *Paramecium* by Chen (1940, *Proc. Nat. Acad. Sci.* V: 26) for *P. bursaria* and this represents the second report of the phenomenon. Pure-line races of the colorless (255) and green (B9) paramecia were mated. The individuals of each race have well-defined micronuclei of approximately equal size.

The three pregamic divisions were found to be remarkably constant in respect to time and micronuclear behavior at a given temperature. However, in the cytological examination of many hundreds of joined pairs, approximately 2 per cent were observed in which the micronuclear behavior resulted in the polyploid conditions only after the pregamic divisions. The crucial stage where polypoidy occurs is found during the period of pronuclear transfer, approximately 16-18 hours after the animals have been mated. It follows the third suggestion made by Chen in accounting for polypoidy; namely, the failure of a migratory pronucleus in one of the conjugants to migrate to the other conjugant. The result is an individual with one small pronucleus (the "stationary") which is haploid, and the conjugant with three pronuclei (two "migratory" and one "stationary") which fuse and form a larger triploid synkaryon.

What is the fate of each nuclear body that is now comparable to the normal synkaryon? The subsequent micronuclear stages show a conspicuous and persisting size difference in all later stages and hence are recognized easily. In the haploid conjugant, late anaphase stages (comparable to postgamic ones) measure 10.8μ in length and are very narrow; similar stages in the triploid co-conjugant measure 27μ in length and are proportionately wider. Their division products measure 8μ in the haploid and 15.5μ in the triploid individuals respectively.

While polypoidy occurs in only 2 per cent of the cases in this material, it nevertheless creates variation in micronuclear composition and is therefore of evolutionary significance.

* Kindly furnished by the Winthrop Chemical Company.

The Lipids in Pelomyxa carolinensis. CHARLES G. WILBER.

In 1942 the author demonstrated that the cytoplasm of *Pelomyxa carolinensis* contains lipid material, that this lipid comes from digested food, and that it is composed of a high proportion of fatty acid. In the latter respect the stored fat differs from that in *Amoeba proteus* in which fat is stored in the neutral form. In the previous work, Nile blue sulfate was used to distinguish neutral fat from fatty acid. This dye has been criticized as a reagent for fat tests. Consequently it seemed desirable to use specific chemical procedures to ascertain the nature of the lipid material in *Pelomyxa*.

Ninety mg. (wet weight) of pelomyxae were thoroughly washed in boiled culture fluid. By repeated centrifugation the cells were broken up and then the lipids were extracted in hot alcohol. The quantities of phospholipid, cholesterol, and fatty acid were ascertained by the Bloor method. It was found that in the amount of cellular material used there was no measurable phospholipid or cholesterol. The total weight of fatty acid in 54 mg. of cells was 2.05 mg. or 3.8 per cent fatty acid.

These results are in agreement with the results previously obtained using Nile blue sulfate. It seems that in *Pelomyxa carolinensis* the lipid material occurs chiefly as fatty acid and that the amount of other lipids is very small.

The presence of lipase in Pelomyxa carolinensis. CHARLES G. WILBER.

The digestion of fat in rhizopods has been demonstrated by several investigators. Moreover, it has been shown that the fats digested are incorporated into the cytoplasm. However, none of the investigations so far has given direct evidence for the presence of lipase in the cytoplasm of rhizopods.

Pelomyxae were starved and then ground up in a drop of water. A drop of this solution was added to a drop of 0.2 per cent emulsion of castor or olive oil and a drop of pure water was added to another drop of the emulsion as a control. After 30 minutes both drops were treated with hydroxylamine hydrochloride and potassium hydroxide. Then after acidification each drop was treated with 1 per cent ferric chloride solution. In each case a violent brown color was produced in the control, whereas no color was produced in the drop containing the ground up pelomyxae.

The above reaction is a test for esters. Lipases are known to be ester ferments. Since the oil emulsions mixed with ground pelomyxae did not give the characteristic ester reaction, it can be concluded that the esters were broken down by something in the cytoplasm. We therefore have direct evidence for the presence of lipase in *Pelomyxa carolinensis*.

PAPERS PRESENTED AT THE MEETING OF THE SOCIETY
OF GENERAL PHYSIOLOGISTS

SEPTEMBER 5 AND SEPTEMBER 6

FIRST SESSION—S. C. BROOKS, CHAIRMAN

The effect of cold on capillary permeability and fluid movement in the frog.
ELLEN BROWN, M.D.* AND EUGENE M. LANDIS, M.D.

Micro-manipulative methods were used to study the relationship between capillary blood pressure and the rate of fluid movement through the walls of single capillaries in the frog's mesentery (a) at ordinary room temperatures of 22.5° to 25.5° C. and (b) when the mesentery was cooled to between -2° and +2° C. Cooling the mesentery decreased capillary permeability, reduced the observed rates of filtration and increased the observed rates of absorption. The filtration constant of the capillary wall was reduced from the control value of 0.0070 $\mu^3/\mu^2/\text{sec.}/\text{cm.}$ water pressure to 0.0019, a decrease of 73 per cent. The effective osmotic pressure of the blood within the capillaries was elevated from 10.5 to 13.8 cm. water, an increase of 31 per cent.

Four possible causes for the increase in apparent or effective osmotic pressure were considered. (1) An increase of *absolute colloid osmotic pressure* was excluded because plasma protein concentrations, calculated from specific gravities of plasma samples, were the same in the two series of frogs. (2) An increase in *effective colloid osmotic pressure* due to greater retention of plasma protein during cooling could also be excluded because the control experiments showed that plasma proteins were already retained completely, or almost completely, even at room temperature. (3) It is possible, however, that the *effective non-protein osmotic pressure* might rise if the passage of smaller molecules, e.g., glucose, amino acids, urea or certain electrolytes, was impeded more than that of water as the permeability of the capillary wall decreased during chilling. (4) *Thermosmosis* might also be responsible because relatively warm blood was circulating through capillaries surrounded by cooler tissues. Studies are in progress to determine whether or not this factor modifies the movement of fluid through membranes *in vitro* using a schema which simulates the conditions existing *in vivo*.

The effects of cold on the capillaries of the frog differ from those observed in mammalian capillaries because the former become less permeable at 2° C., whereas the latter become more permeable as temperature falls below 10° C. However, actual freezing of the frog's capillaries at temperatures of -5° to -10° C. increased capillary permeability conspicuously, as shown by the appearance of stasis during thawing. If the duration of actual freezing was brief this stasis usually disappeared within a few minutes as the capillary wall regained its normal relative impermeability to protein.

Bubble formation within single cells.† E. NEWTON HARVEY, K. W. COOPER, A. H. WHITELEY, D. C. PEASE, AND W. D. McELROY.

Normal living isolated cells (*Amoeba*, sp., *Chaos chaos*, *Paramoecium*, *Arbacia* and *Asterias* eggs and *Nitella*) do not form internal gas bubbles if saturated with nitrogen gas at 80 to 120 atmospheres pressure and then suddenly decompressed. Bubbles may form on the outside of the cells due to contamination with gas nuclei (minute gas phases sticking to hydrophobic spots). Cells which have been killed by chloroform or formalin likewise form no bubbles within but sometimes spontaneously dead cells or those previously injured by twisting before subjec-

* Research Fellow in Physiology, Commonwealth Fund.

† Part of the work described in this Abstract was done under a contract recommended by the Committee on Medical Research between the Office of Research and Development and Princeton University.

tion to the high gas pressures do form bubbles within after decompression. A living *Nitella* cell just after decompression from high gas pressures and still free of bubbles, will immediately form a bubble inside if the cell wall is gently pinched (not enough to penetrate the wall) or twisted. Such bubbles are believed to result from local decreased tensions that tear the liquid, forming a space or cavity (a vapor phase) into which gas diffuses, forming a gas nucleus that immediately grows to a bubble. Such cavities can form inside or outside of cells even at atmospheric pressures. They are believed to be formed during muscular exercise in man, when the incidence of aviator's bends is greatly increased.

The action of various cations of muscle protoplasm. L. V. HEILBRUNN AND F. J. WIERCINSKI.

There are two ways to study the colloidal behavior of muscle protoplasm. One way is to isolate pure proteins and follow their reactions in test tubes; the other way is to subject the protoplasm itself to reagents and observe the results. In our studies, we injected solutions of various salts into the interior of isolated muscle fibers of the frog. We then noted the degree of shortening of the constituents of the muscle. With the aid of a micrometer eyepiece, we were able to determine the effect of the injections on the length of the fiber. In numerous experiments we found that rather dilute calcium chloride solutions invariably caused an immediate and pronounced shortening of the protoplasmic constituents of the muscle. On the other hand, potassium and sodium chloride had very little effect. Even when injected in concentrations isotonic with the muscle, they ordinarily caused no shortening whatsoever. Rarely, a shortening did follow injection of isotonic sodium or potassium chloride. This we believe was due to the release of calcium ion. Magnesium ion likewise causes no shortening of the protoplasmic constituents. Barium acts like calcium. The results support the calcium ion theory of stimulation and they are opposed to Szent-Györgyi's opinion that potassium is the ion primarily responsible for the contraction of muscle.

Further observations on an oligodynamic action of copper and mercury on erythrocytes. M. H. JACOBS AND DOROTHY R. STEWART.

The specific effect of copper in decreasing the permeability of erythrocytes to glycerol seems to be absent in all species whose erythrocytes show a low degree of permeability to this solute. It is also lacking in a number which show a very high permeability both to glycerol and to other hydrophilic solutes of comparable molecular volume. It has so far been found only in those species whose erythrocytes show a disproportionately great permeability to glycerol, thus suggesting that some special mechanism of penetration may be involved, which is reversibly inactivated by copper. This generalization is supported by the behavior of the erythrocytes of a number of birds in which the specific permeability to glycerol is particularly great.

The effects of HgCl_2 in some ways resemble and in others differ from those of CuCl_2 . One of the most important differences is that HgCl_2 forms a double salt with NaCl , and its activity is therefore greatly reduced by the presence of any considerable quantities of the latter salt. A second difference is that HgCl_2 readily enters the erythrocyte, while CuCl_2 does not. These two fundamental differences are responsible for a number of secondary ones.

That copper may hinder the escape of glycerol from human erythrocytes, as well as its entrance into them, is suggested by the following experiment. To a suspension of erythrocytes in an isotonic salt solution, small amounts of copper and of concentrated glycerol are added, and the resulting mixture is then diluted with a properly chosen hypotonic salt solution. If the copper is added before the glycerol, it decreases hemolysis by preventing the entrance of glycerol into the cells. If the copper is added 30 seconds or more after the glycerol, it increases hemolysis by preventing the escape of the glycerol that has entered the cells.

The prolytic loss of K from red cells. ERIC PONDER.

The prolytic loss of K, i.e., the loss of K which takes place from red cells exposed to hypolytic concentrations of lysin, has been measured by means of the flame photometer in systems containing distearyl lecithin, sodium taurocholate, sodium tetradecyl sulfate, saponin, and digitonin. The

lysins are added in various concentrations to washed red cells from heparinized human blood, and the K in the supernatant fluids is determined after various intervals of time at various temperatures. This prolytic loss of K , K_p , is compared in every experiment with the loss K , into standard systems containing one per cent NaCl alone, without lysin.

The losses K_p and K , increase with time, so that new steady states are approached logarithmically. The values of K_p which correspond to the new steady state depends on the lysin used, being greatest with taurocholate and smallest with powerful lysins such as digitonin (confirming an observation of Davson and Danielli). The extent and course of the K losses seem to have no simple relation to the prolytic phenomenon of the disk-sphere transformation.

Just as the prolytic loss of K occurs without the loss of any Hb, so in concentrations of lysin sufficient to produce hemolysis the loss of K , expressed as a percentage of the total red cell K , increases much more rapidly with lysin concentration than does the loss of Hb, expressed as a percentage of the total Hb. The explanation of these relations depends on whether the loss of K is treated as being all-or-none in the case of the individual cell, or as being the result of the loss of part of the K from all the cells. This point has yet to be decided.

SECOND SESSION—I. MICHAELIS, CHAIRMAN

Effect of fluoroacetate on the metabolism of baker's yeast. E. S. GUZMAN BARRON AND GEORGE KALNITSKY.

Among the organic halogen compounds, those containing fluorine occupy a special position regarding their chemical and physiological properties. Because of the high value of the energy of the C-F bond and of the electro-negativity of F, the introduction of F into the C atom produces a greater stability, specially in aliphatic compounds. This is shown on measuring the rate of combination of cysteine with halogen acetates. At 23°, half-reaction with iodoacetate took place in 4.4 minutes; with bromoacetate in 6.2 minutes; with chloroacetate, in 125 minutes. With fluoroacetate it did not react at all. There is a certain relationship between the rates of reaction and the bond-energy values of the C-halogen bonds as well as the electronegativity values of the halogens. On studying the effect of these halogen acids on the rate of oxidation of acetate by baker's yeast it was found that 0.001 M of fluoroacetate inhibited it 90 per cent; bromoacetate, 17 per cent; iodoacetate, chloroacetate, and trifluoroacetate, none at all. On comparing the interatomic distances between C and the halogen it can be seen that the C-F bond with a distance of 1.41 Å approaches most closely the distance of the C-H bond, 1.09 Å. By increasing the size of the fluoroacetate molecule through the replacement of the other two hydrogens with fluorine (trifluoroacetate) the inhibiting effect was destroyed. This inhibition is a substrate competitive inhibition, the fluoroacetate occupying the place of acetate in the protein moiety of the acetate metabolism enzyme. Increase of the length of the molecule as in fluoropropionate, fluorobutyrate, and fluorocrotonate destroyed the inhibition. Inhibition was partially reversed on addition of large amounts of acetate (0.08 M). Inhibition occurs in the first step of acetate metabolism, namely, condensation with oxaloacetate to give citrate. The formation of citrate from acetate was completely inhibited with 0.005 M fluoroacetate. In the presence of ethanol, the rate of O_2 uptake was not affected by fluoroacetate up to 42 per cent of the total O_2 uptake, when the inhibitory effect appeared. This is indication that ethanol oxidation occurs in three successive steps: oxidation of ethanol to aldehyde; and of aldehyde to acetate, both unaffected by fluoroacetate; and oxidation of acetate, inhibited by it. At the end of the experiment there were in the control 1572 cmm. O_2 used, and 150 cmm. of acetate formed from 940 cmm. of ethanol; in the presence of fluoroacetate there were 975 cmm. O_2 used and 890 cmm. of acetate formed.

The effect of sodium azide on Paramecium calkinsi. E. J. BOELL.

Sodium azide is generally regarded as an inhibitor of respiration by virtue of its inactivation of cytochrome oxidase. In *Paramecium calkinsi*, experiments have shown that this compound, in a concentration of 0.001 to 0.01 molar, reversibly depresses respiratory activity by 50 to 60 per cent. Under certain circumstances, however, azide instead of inhibiting respiration serves as a powerful respiratory stimulant. The stimulating effect of azide seems to depend primarily upon the pH of the medium. For example, a 0.01 molar solution of NaN_3 at pH 6.02 will depress respiration to a value about 30 per cent of normal; at pH 6.24 respiration is 70 per cent of nor-

mal, while at pH 6.59 the same concentration of azide stimulates respiration of 238 per cent of normal. Calculation of the hydrazoic acid concentration at these pH values shows that the effect produced depends, within certain limits, upon the concentration of undissociated HN_3 .

A study has been made of the mechanism of azide stimulation. It has been found that the respiratory quotient of normal animals averages 0.99; that of animals in the presence of a stimulating dose of azide averages 1.05. The increased oxidation thus involves the metabolism of organic substrate. It is also apparently mediated by the normal enzymic mechanisms for it is sensitive to cyanide. Carbon monoxide, however, exerts only a slightly depressing effect.

The metabolism of *Paramecia* under normal circumstances is accompanied by the production of large quantities of ammonia nitrogen. On the assumption that such ammonia production represents protein breakdown, approximately 75 per cent of the total oxygen consumption of control animals can be accounted for in this way. Although *Paramecia* treated with azide show increased ammonia production, only 22 per cent of the extra oxygen uptake induced by azide can be accounted for as protein breakdown with ammonia as the end product.

In addition to the effects already noted, azide interferes with the ability of *Paramecium calkinsi* to maintain normal water balance. The activity of the contractile vacuoles is greatly reduced and supernumerary vacuoles are frequently formed.

The oxygen consumption concerned with growth in bacterium coli. KENNETH FISHER. No abstract submitted.

Enzymatic acetylation and the coenzyme of acetylation. FRITZ LIPMANN.

The mechanism of enzymatic acetylation of aromatic amines has been studied in pigeon liver homogenates and extracts (Lipmann, F., 1945. *J. Biol. Chem.*, 160: 173). In this enzymatic system the condensation of an aromatic amine, like sulfanilamide, with acetate, is effected through a transfer of phosphate bond energy from adenylylphosphate. (Cf., Nachmansohn, D. and Machado, A. L., 1943. *J. Neurophysiology*, 6: 397 for a similar system of choline acetylation in brain).

A heat stable and dialysable coenzyme was recently found necessary in this reaction, besides the energy donor adenylylphosphate. The characterization of this new coenzyme is now in progress in this laboratory in collaboration with Dr. Nathan O. Kaplan. We find the same coenzyme necessary to complement dialyzed brain extracts for acetylation of choline, although the brain enzyme is specific for choline and the liver enzyme specific for amines. The coenzyme is present in largest amounts in brain, liver, and kidney. Appreciable amounts are present in all tissues tested, including carcinoma. Therefore its action must be a very general one and probably not merely restricted to acetylation.

The coenzyme is destroyed by intestinal phosphatase with liberation of phosphate. It is inactivated by a rather general tissue enzyme without liberation of phosphate. The link attacked by the latter enzyme is unknown. The compound follows the general pattern of nucleotide precipitation. Our most active preparations showed sporadic crystals on microscopic examination. This quite uniform fraction contained adenine, ribose, and phosphate in the proportion 1 to 1 to 2. Acid hydrolysis showed the second phosphate not to be in pyrophosphate linkage. If we assume the presence of some crystals to indicate near purity, which it not necessarily does, then the content of approximately 50 per cent of adenylic acid in our best preparations should mean the coenzyme to be of dinucleotide structure. Therein the adenylic acid should be linked through the second phosphate to an as yet unidentified part. Electrotitration and cleavage experiments seem to support the outlined constitution.

Penetration and action of cholinesterase inhibitors. DAVID NACHANSOHN. No abstract submitted.

The metamorphosis of visual systems in amphibia. GEORGE WALD.

In the rods of the vertebrate retina two visual systems are found. One is based upon the red photosensitive pigment rhodopsin, engaged in a cycle with vitamin A_1 ; the other involves the purple photopigment, porphyropsin, bound in a similar cycle with vitamin A_2 .

The porphyropsin system appears to be the more primitive in vertebrate evolution. The cyclostome, *Petromyzon marinus*, possesses only this system. The same is true of all types of freshwater fish so far examined.

Vertebrates have followed two pathways out of fresh water, one into the sea, the other to land. Both have led them to the use of vitamin A₁ in vision. Thus all marine fishes which have been examined, with the single exception of certain Labridae, have the rhodopsin system alone; so also do all the birds and mammals investigated.

Interpolated between freshwater and marine fishes are euryhaline forms, which can exist as adults in either environment. Among them, the salmon and the "freshwater" eel have mixtures of the rhodopsin and porphyropsin systems; while the alewife and white perch have only the latter. In all these forms the visual system is predominantly or exclusively that normally associated with the environment in which the fish develops embryonically, and is relatively independent of the environment in which it is found as an adult.

Interpolated between freshwater fishes and true land vertebrates are the amphibia. Their life histories for the most part are closely analogous with those of euryhaline fishes, amphibian migrations to land replacing fish migrations into the sea.

Adult frogs possess the rhodopsin system and vitamin A₁ alone. The tadpole of the common bullfrog, *Rana catesbeiana*, however, has exclusively the porphyropsin-vitamin A₂ system just prior to metamorphosis. During metamorphosis it transfers completely to the rhodopsin system, which is found alone in the new emerged frog. Partly metamorphosed animals have mixtures of both systems, such as have been found otherwise only in euryhaline fishes.

The common newt, *Triturus viridescens*, begins its life as a gilled larva in fresh water. After several months it metamorphoses to the land-living red eft; then after 1-2 years of growth it undergoes a second metamorphosis to the sexually mature newt, returning to the water for the remainder of its life. The eye of the red eft contains a mixture of vitamins A₁ and A₂, predominantly the former; while that of the water-phase adult presents just the reverse proportions of both vitamins. This is a change opposite in direction to that in the frog, but associated in the same way with the chemical metamorphosis of visual systems.

Amphibia, therefore, like euryhaline fishes possess as a group both the rhodopsin and porphyropsin systems; but in amphibia these systems succeed one another as the animal goes through its basic metamorphoses.

THIRD SESSION—J. H. BODINE, CHAIRMAN

X-ray effects in mixtures of compounds. RUBERT S. ANDERSON.

It has been reported previously that ascorbic acid, as shown by experiments in plasma, has a preferential ability to react with the materials produced in water by x-rays. Much of the ascorbic acid reaction is not observed in irradiated muscle. Non-uniform distribution of the ascorbic acid in muscle would tend to make the observed result too low. Another possibility is that other compounds are present in muscle which take some of the reactive material away from the ascorbic acid.

Evidence has been obtained that the ascorbic acid reaction consists in part of a reversible oxidation, presumably to dehydroascorbic acid. When present during the irradiation, glutathione and cysteine gave substantial, although variable, protection of ascorbic acid against x-rays. Alanine was much less effective, suggesting that the sulphydryl grouping is largely responsible, whether it is a true competitive protection or a reversal of oxidation. Glutathione and cysteine and possibly protein sulphydryl groups could thus account for a part of the protective effect of muscle on ascorbic acid.

There is no evidence that the destruction of a small amount of these compounds would damage a cell. However, the work shows that, in principle, a compound through which the water reaction might damage the cell could exist.

Ascorbic acid, glutathione and cysteine partially protect pepsin from the inactivating effect of x-rays. Alanine is much less active.

If at least a part of the reaction in water is distributed randomly throughout the proteins of the cell and nucleus, the occasional loss of a molecule or two from compounds represented by hundreds of molecules need have little effect on the cell although the products formed, such as denatured proteins, might secondarily be harmful to the cell. However, if there are in the

cell or nucleus some thousands of different protein compounds or structures each form of which is essential to the cell and each one of which is represented by but one or two molecules or particles, then randomly distributed products of irradiated water might destroy one of these entities and so damage or kill the cell. This is essentially the argument used by Lea in reaching the conclusion that his theory led to the expectation of gene and chromosomal effects from irradiation.

Electrical studies of acetylcholine and choline esterase. T. C. BARNES.

Acetylcholine passes through the thin oil layer of a bubble of guaiacol-resin-cholesterol so rapidly that the spike potential must be recorded by an oscillograph. First the acetylcholine produces a negative phase boundary potential on one side of the oil layer but on reaching the opposite side of the oil a new potential is established which produces the descending part of the spike (no esterase required). At the suggestion of Osterhout, solutions were shaken 5 hrs. with guaiacol with these results: oil with saline 5×10^{-4} mhos; same with 0.002 M acetylcholine 35×10^{-4} mhos (conductivity was determined of oil separated from aqueous solution). Applying the Nernst equation, 0.058 times log conductivity difference (6) gives 40 mv. (observed phase boundary potential). At the suggestion of Loewi, tetramethylammonium iodide was found to give no potential on nitrobenzene but 0.05 per cent gave 25 mv. negative on guaiacol. The type of oil and not the tertiary or quaternary nature of the compound determines electrogenic effects. Thus prostigmine produces 85 mv. negative on guaiacol compared with 35 mv. generated by acetylcholine (both 0.002 M). Prostigmine inhibits the cord in spasticity by flooding with high negative potential which may also act as a stimulus on muscle in myasthenia. Dialantin and phenobarbital produce positive potential (20 mv. at concentration of 0.05 per cent) which probably neutralizes the excess negativity of acetylcholine in the brain in epilepsy. Lyovac plasma reduces the phase boundary potential of 0.05 per cent acetylcholine from 35 to 15 mv. (residual potential is produced by choline). Potential of benzyol choline is destroyed by serum and part of the mecholyl potential by one per cent ground cat brain. Eserine and DFP preserve the potential of acetylcholine in the oil-cell in the same manner as in the nerve.

One per cent DFP increases the specific conductance of guaiacol 100 per cent which explains part of its blocking action on nerve and muscle.

The action-current in cholinergic nerve is probably a phase-boundary potential of acetylcholine (sympathin is the electrogenic amine in adrenergic nerve).

Two schools of thought in electrophysiological theory. R. BEUTNER AND T. C. BARNES.

The older school, entrenched as the hypothesis of sieve membranes retaining negative but not positive ions, explains everything but solves no problems. The newer school omits hypotheses and proposes searches for electrogenic materials in tissues by setting up artificial battery systems composed of lipid layers (oils) inserted between aqueous salt solutions. Some of these resemble analogous battery systems containing a tissue in place of the lipid. One type of system studied is:—concentrated saline/tissue or lipid (oil)/diluted saline +. Tissue, in such a set-up, may produce the maximum e.m.f. of 58 millivolts if the concentrated solution is 1/10 mol.; the diluted one, 1/100 mol. Only few oils show such an effect, as e.g., fatty acid dissolved in a phenol-derivative, but not neutral fats, gelatin, etc.

The production of bio-electricity does not depend on such aqueous salt concentrations but on metabolic processes in tissues, chiefly oxidation. A search for suitable electrogenic systems has led to the following one (Beutner, Loznere, 1930):—saline/reduced substance e.g., a higher alcohol or lower fatty acid as in dying tissue/oxidized substance e.g., corresponding acid or corresponding higher fatty acid as in respiring tissue saline +. For the action current one possible electrogenic substance is acetylcholine since even dilute solutions produce an e.m.f. in contact with oils in a system such as: + saline without addition/oil/saline with acetylcholine added 1:100,000 to 1: several million—(Beutner and Barnes, 1941).

Tissue extract can be used in the place of the oil, also frog's nerve by the Netter technique. Adrenergic amines produce similar negative potentials but on different oils which are inactive in contact with choline esters. A difference in chemical composition may therefore be responsible for the specific function of cholinergic and adrenergic fibers. The rapid disappearance of the

negative potential, which occurs even in the absence of choline esterase, may be explained by a penetration of acetylcholine through a thin lipoid layer (membrane) creating a potential difference in the opposite direction on the other side. Physico-chemical studies are not needed for the search for electrogenic substances, but when performed on oil cells, they show the existence of phase boundary potentials depending on electrolyte distribution; the charged pore theory fails to explain the phenomena and is contradictory.

The frequency of x-ray-induced chromatid breaks in Tradescantia as modified by near infrared radiation. C. P. SWANSON AND ALEXANDER HOLLAENDER.

The frequency of x-ray-induced chromatid breaks in *Tradescantia* can be significantly increased by treatment of the inflorescences with near infrared radiation. Pretreatment with near infrared radiation for seven hours prior to x-radiation increased the frequency of single deletions, double (isochromatid) deletions, and translocations between and within chromosomes; post-treatment increased only single deletions and translocations. A delay of 21 hours between treatment with infrared and x-rays did not appreciably decrease the effectiveness of the infrared, suggesting that the changes within the cell induced by the infrared were of a relatively permanent nature. At the present time, the nature of the effect of infrared is not clearly understood.

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A STRONGLY INTERSEXUAL FEMALE IN *HABROBRACON*

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In the parasitic wasp *Habrobracon juglandis* (Ashmead), diploid males have never shown any tendency toward intersexuality; they are as definitely male as their normal haploid brothers. When a "diploid male with female genitalia" was found, it was therefore regarded with especial interest. The specimen, designated freak 994, developing from a heavily x-rayed (29,300 r) egg, occurred among the offspring of a treated wild type (stock 33) female crossed with an untreated lemon honey male (Experiment by A. R. Whiting, 1945).

Freak 994 shows the heterozygous condition of the semidominant body color gene lemon inherited from its father. (Note light base of antennae in Figure 1.) The number of its antennal segments and its large ocelli are male characteristics. It was to be expected, therefore, that male reproductive reactions would occur. Several tests at different times failed to evince any response toward females although the specimen appeared healthy, drank honey water and lived for several days until fixed in Carnoy fluid. Since it likewise failed to give any response (female) to caterpillars, its indifference was probably not due to its sex type but to some unknown factor.

Because of the small "feminized" genitalia on the "male" body, freak 994 was at first recorded as a "diploid gynandroid male." Gynandroids, however, have always been haploids. They are mosaic males in which the two sexually different types of male tissue react in a complementary way to feminize the external genitalia (Whiting, Greb, and Speicher, 1934). Their mosaicism is shown by their asymmetry, not only in body color, in number of antennal segments, in mutant traits, and often in wing length, but especially in the external genitalia which are a mixture of normal male and feminized male structures with much reduplication and irregularity. In freak 994 there are no male genital structures and the female genitalia, consisting of a pair of sensory gonapophyses with no visible sting, are symmetrical and larger than in gynandroids. They are much smaller, however, than the female genitalia found in gynanders which are male-female mosaics with clearly separated male and female regions. That freak 994 is not a sex mosaic is shown by its symmetry in body coloration, in antennal flagella with nineteen segments in each and in length of wings and legs.

Two types of intersexes have hitherto been reported in *Habrobracon*. (1) Gynoid, dependent upon a single mutant gene, is a weakly intersexual male, functioning normally as a male, but having certain external traits, including antennae, feminized. (2) Nine intersexual females were reported (Whiting, 1943) occurring in a single fraternity. "Superficially, these appear to be the reverse of the gynoid

males, being more masculine anteriorly, feminine posteriorly." They resemble freak 994 in head and thorax and in the anterior part of the abdomen which are altogether like those of the male. In the posterior region, however, the sclerites are thickened, there is a normal sting and the sensory reproductive appendages are of full length characteristic of the female. "The nine intersexual females must be



FIGURE 1

regarded as more strongly intersexual than gynoid males since antennae, ocelli and instincts are completely sex reversed." Freak 994 is an intersexual female, comparable to these nine but still more strongly intersexual because of greater restriction of the "female" region and reduction of the genitalia.

In *Habrobracon*, normal haploid males have cells almost as large as the corresponding cells of diploid females and in some stocks they are actually larger (Grosch,

1945). Cells of diploid males are much larger than are those of females or of haploid males. These relationships have been determined by counts of microchaetae within a given area on the upper surface of the wings, each microchaeta corresponding to a single cell. Study of the dispersion of microchaetae in freak 994 showed its cell size to be within the range for the female or haploid male and therefore much smaller than that characteristic of the diploid male. The marked shift of the intersex in the male direction does not then affect the size of its cells. It may be fundamentally female, heterozygous for the sex factor. This condition perhaps prevents the abnormal expansion of cell size while permitting development of antennae and ocelli of normal male type.

The nine intersexual females previously reported had internal abdominal structures as in the female with normal poison sac and glands and seminal receptacle. Each ovary, however, appeared to be a pair of sacs of oogonia showing no differentiation of nurse cells and ova. Serial sections were made of the abdomen of freak 994 and the internal structures were studied. The digestive tract is entirely normal with the crop greatly distended from honey water feeding. A poison apparatus is present but imperfectly developed and situated near the median plane, directly dorsal to the compound posterior nerve ganglion instead of being shifted laterad to the digestive tract. The poison glands are normal although of somewhat small size. Their ducts converge to a common duct connecting distally with an imperfect poison "sac" and proximally traversing the very short distance to the region where normally lies the root of the sting. The poison "sac," of approximately normal length, is reduced in diameter to an irregularly sclerotized strand. It is surrounded by longitudinal muscles as in a normal female. Nothing corresponding to a seminal receptacle could be located, nor were any gonads to be found. The fat body appears normal, surrounding the digestive tract and the poison apparatus dorsally and laterally.

DISCUSSION

In the report on the nine intersexual females, it was suggested that they might be accounted for by a dominant mutation in a sex allele changing xb to xb^m . The intersexes would then be modified females, xa/xb^m . A similar hypothesis would cover freak 994, but here the mutation may have been x-ray induced and more potent than in the previous case so that the intersexuality would be more extreme with turning-point earlier in development.

Failure to find gonads in freak 994 does not necessarily mean that they were lacking from the beginning for they may have begun development and then disintegrated.

Comparison may be made between freak 994 and certain types of "deficient" individuals previously reported in *Habrobracon* (Whiting, 1926). Some of the "deficient" had external genitalia lacking but gonads present. Others had testes of reduced size, or present on one side, lacking on the other. Some of the "deficient" females with no trace of poison apparatus had well differentiated ovaries with eggs and nurse cells. This is just opposite to the condition found in the intersexual female, freak 994. There was no intersexuality among the "deficient."

SUMMARY

An intersexual female developed from a heavily x-rayed egg fertilized by an untreated sperm. The specimen is more strongly intersexual than a group of nine previously reported, for its external female genitalia are much reduced, its poison apparatus defective and its ovaries altogether lacking. Externally, it appears like a diploid male with small female genitalia.

It is suggested that the x-radiation may have caused a change within a sex-differentiating allele, so that the heterozygote would develop into an intersex rather than a normal female.

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LOCI OF ACTION OF DDT IN THE COCKROACH (PERIPLANETA AMERICANA)

J. M. TOBIAS AND J. J. KOLLROS*

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In the cockroach, DDT produces symptoms which clearly reflect involvement of the neuromuscular apparatus. These are qualitatively much the same in all arthropods which have been studied, though there are important quantitative differences. Thus, in any given animal the time course of the poisoning is a function of dose, and for a dose of comparable toxicity in terms of final mortality, the symptoms unfold and death occurs much more rapidly in some insects (the fly) than in others (the roach) (Tobias, Kollros, and Savit, 1946a). In the roach, the sequence of symptoms is initiated by hyperextension of the legs, elevation of the center of gravity and development of postural instability. The hyperextension then decreases and is superseded by increasing and generalized tremulousness, involving the head, body, and all appendages; the gait becomes ataxic, and minor stimuli of sound or touch result in great hyperactivity, exhibited mainly in running and climbing. The animal falls on its back time after time until finally it can no longer right itself. Leg movements continue in the supine insect with two components, a high frequency intermittent tremulousness and a slower incoordinated flexion and extension. These two types of activity possibly reflect the double innervation which has been described for cockroach muscle (Pringle, 1939), one fiber type producing relatively slow tonic contractions, the other producing relatively fast twitches. It will be seen later that after poisoning these two types of movement can be independently altered. Activity finally diminishes progressively. The fast tremors disappear first and finally there remain only occasional isolated movements of body wall, tarsi, palpi, cerci, or antennae. When no further somatic movement can be detected, the heart usually continues to beat for some time, and electrical stimulation of the nerve cord may still evoke muscle responses. The animal may live in this condition for a day or so and finally die.

Mammals exhibit a similar symptomatology up to a point. In the rat and dog, given DDT intravenously or orally, muscular fibrillations and excessive blinking are followed by tremulousness, ataxia, falling and gross convulsive seizures. The animal may have a number of convulsions and die in the tonic phase of one or recover after gradual subsidence of symptoms. There is no period of prostration and nearly complete immobility as in the insect, because death occurs when systematic respiratory movements cease. In the insect, the small amount of body movement and twitching sufficiently augment diffusion for respiratory exchange. Then too, the insect is far more resistant to anoxia than is the mammal (Wigglesworth, 1939).

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The frog, as might be expected, responds more like the insect than the mammal (Tobias, Kollros, and Savit, 1946b). Respiratory exchange through the skin can sustain life, and, after a period of hyperirritability, the animal lies prostrate and more or less immobile. Such symptomatology has prompted a number of investigations designed to discover a locus of action of DDT. As will be seen, there probably are a number of sites of action depending largely on dosage, but this point of view was only gradually attained.

In mammals (Crescitelli and Gilman, 1946), DDT apparently does not act directly on either muscle, myoneural junction or spinal cord. Since tremors persist after decerebration and mesencephalic transection, and since abnormal cerebral and cerebellar electrical activity persists after atlanto-occipital transection, neither cerebral cortex nor basal ganglia can be a critical site of action, and intact spinal afferents are obviously not necessary for the central effect. The cerebellum is considered, by these authors to be the most likely critical site of action in the mammal. Locus of action has also been investigated in insects. In *Drosophila* (Bodenstein, 1946), DDT seems not to act on muscle or myoneural junction, but does act on peripheral nerve and may act on the central nervous system. In the cockroach (*Periplaneta americana*), DDT has been found to act on nerve in high concentrations (Yeager and Munson, 1945), and, in low concentrations, on peripheral receptors (probably proprioceptors) (Roeder and Weiant, 1946). The latter workers also have evidence which they interpret to mean that high concentrations may act directly on either the myoneural junction or muscle itself. In the crab (*Cancer irroratus*) there is evidence for action on motor nerves (Welsh, 1946).

It was the purpose of this study to further investigate loci of action of DDT in an insect. Because of its large size and ready availability, the cockroach (*Periplaneta americana*) was used throughout.

METHODS

Cockroaches were immobilized by exposure to 100 per cent CO_2 for 20–60 seconds or by etherization. After CO_2 , anesthesia seldom lasted over a minute. Once anesthetized, the roach was fastened to a bit of cardboard by pins passed through either side of the pronotum. Appendages were held in any desired position by pins crossed over the body.

Decapitation was easily achieved by simply cutting the neck with a small scissors. The exposed stump was sealed with low melting-point paraffin. Ligation of the neck prior to decapitation to prevent loss of hemolymph did not prolong survival time. Such animals live about 60 hours (Table I).

To expose a thoracic ganglion, the spinasternum just caudal to the ganglion was cut through, and the incision extended along the sides of the sternal plate. After the plate was reflected forward, removal of superficial fatty tissue and tracheal tubes fully exposed the ganglion. The connectives anterior to the ganglion were held in a jeweler's forceps and cut with iridectomy scissors. Traction on the connectives exposed the lateral nerves, which were sectioned. Finally, the posterior connectives were cut and the ganglion removed. Simple isolation of a ganglion from the rest of the nerve cord can be achieved without excising it by cutting the connectives through slits in the cuticle. Complete transection of the entire roach between sets

TABLE I

Effect of lesions of the central nervous system on symptoms of DDT poisoning in the cockroach

Operation		No. roaches			Average survival, hours	General result-	Legs which showed DDT effects
		Controls, no DDT	Operated				
Nature	Site (See Fig. 1)		Before DDT	After DDT	Hyperactivity, tremors and convulsions		
Decapitation	At A	14	—	—	61	Rare tremors in 3 animals—no convulsive activity	None
		—	14	—	56	Typical DDT effects in all animals	VI
		—	—	14	56	Typical DDT effects in all animals	VII
Transaction of ventral nerve cord	At C and D (both anterior and posterior to thoracic ganglion no. 2)	16	—	—	104	None in any animals	None
		—	15	—	60	Typical DDT effects in all animals	All in 13 animals; 2nd and 3rd pairs in two animals
Destruction of ganglion	Th. 2 (thoracic ganglion no. 2)	15	—	—	77	None in any animals	Leg 2 paralyzed in all
		—	15	—	71	Typical DDT effects in all animals	1 and 3 in 13 animals. 1, 2 and 3 in two animals

of legs results in an isolated segment containing a ganglion, nerves and the attached legs. Such a preparation, if kept moist, is viable for at least 6 to 8 hours.

Excision of the heart largely prevents circulatory removal of substances applied to structures to elicit a local effect. Longitudinal incisions through the cuticle, on either side of the heart tube along its entire length, isolate a strip whose removal carries the heart with it. The heart may be cauterized with equal ease (Yeager and Munson, 1945).

Methods for the administration of measured doses of DDT to insects have been described elsewhere (Tobias, Kollros, and Savit, 1946a).

RESULTS

Localization experiments with uncontrolled DDT doses

Except where otherwise specified, contact poisoning was carried out by confining the roach for 5–15 minutes within a glass cylinder coated with DDT previously precipitated from acetone solution.

Roaches decapitated before or after such contact with DDT behaved like intact poisoned animals (Table I). Therefore, neither the supra- nor the sub-oesophageal ganglia are essential for the development or maintenance of DDT-induced motor activity in the legs or body. Ventral nerve cord connectives were transected both anterior and posterior to the mesothoracic ganglion (Fig. 1, levels C and D). Animals so prepared but given no DDT showed incoordination of the mesothoracic legs when walking, but there were no symptoms which could be confused with those of DDT poisoning. When such animals were subsequently poisoned, however, the mesothoracic as well as the other legs exhibited typical abnormal activity (Table I). After complete transection of the whole body of the poisoned roach, at both these levels (excised segment Fig. 1), leg tremulousness and hyperactivity continued un-

abated in the isolated segment. The application of DDT emulsion or DDT in acetone to the cut surface of such segments obtained from normal roaches evoked typical DDT effects in the attached legs within a few minutes. The same was true of DDT applied directly to the exposed ganglion in the otherwise intact animal. Emulsion or acetone without DDT had no such effect.

The cells of origin of the leg nerves lie within the lateral halves of the thoracic ganglia, each ganglion in the adult being formed by the midline fusion of two embryonic ganglion masses. Median sagittal section of the ganglion in a poisoned roach (Fig. 1, level F) did not stop hyperactivity in either of the legs innervated

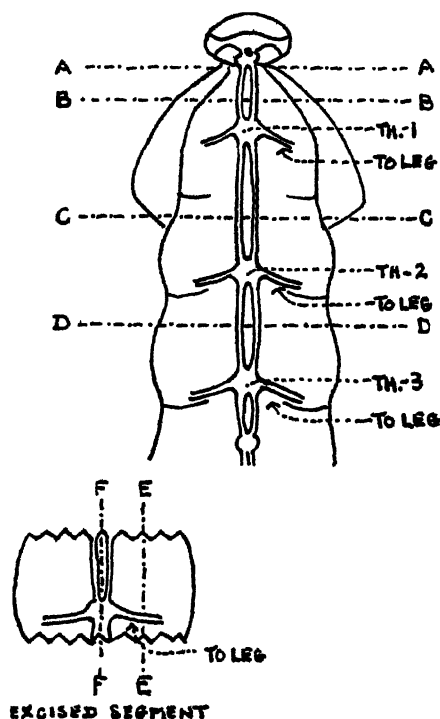


FIGURE 1. Levels of section in cockroach nervous system

from the resulting ganglion halves. Therefore, even half a segment contains all the structures necessary for the maintenance of DDT symptoms in a leg.

If, however, the entire ganglion was removed the results were generally quite different. Mesothoracic ganglia were removed from thirty normal roaches. The corresponding legs of all were paralyzed and failed to respond to touch or pressure. Shortly after the operation, fifteen of the animals were contact poisoned. All showed typical DDT effects in the pro- and metathoracic legs, but the ganglionectomized mesothoracic legs remained entirely quiet in thirteen and showed only occasional tarsal twitching and some slight movement of the other joints in two. Similarly, ganglionectomy after the development of hyperactivity, rather than before

TABLE II

Experiments on isolated roach segments containing local ganglion, nerves, and leg

No. of segment-	Material applied	Route	Number of segments in which there was persistent DDT leg activity
6	Nothing	—	None
3	Emulsion* without DDT	On cut surface	None
2	Acetone without DDT	Injected	None
7	1 Per cent DDT emulsion*	On ganglion	Occurred in all
4	10 Per cent DDT in acetone	Injected into vicinity of ganglion	Occurred in all
3	DDT powder	On ganglion	Occurred questionably in one

* Emulsion 1 per cent DDT, 10 per cent peanut oil, 1 per cent lecithin and 88 per cent 0.90 per cent NaCl (5).

poisoning, either stopped or markedly reduced symptoms in the corresponding legs (Table IV). As was to be expected from these experiments, section of leg nerves lateral to the ganglion stopped or markedly reduced activity in many (65 per cent) of the legs (Table III).

These experiments tentatively suggested that the ventral cord ganglion was critically involved in the motor action of DDT and might itself be a site of action. Conflicting data, however, were also obtained. It was possible, as also reported by others (Yeager and Munson, 1945; Roeder and Weiant, 1946), to produce motor

TABLE III

*Visible effect of DDT on amputated legs
(Dose not controlled)*

No. of legs	Source of legs	Treatment after amputation	Results after amputation
30	Normal roaches	Normal controls	No spontaneous movement
58	DDT poisoned roaches tremulous and hyperactive	—	Continued activity in 20. No movement in 38
12	Normal roaches	Emulsion without DDT injected into cut end	No movement in any
22	Normal roaches	Emulsion with 1 per cent DDT applied to cut end	Movement in 1, others all quiet
35	Normal roaches	Emulsion with 1 per cent DDT injected into cut end	Movement in 25, other 10 quiet
9	Normal roaches	Acetone without DDT injected into cut end	Movement in 1, other 8 quiet
13	Normal roaches	Acetone with DDT injected into cut end	Movement in 3, other 10 quiet

activity in a large percentage of amputated legs by the injection of DDT emulsion (1 per cent DDT, 1 per cent lecithin, 10 per cent peanut oil, and 88 per cent 0.9 per cent NaCl solution). It will also be recalled that ganglionectomy failed to entirely quiet the legs in two of fifteen experiments (Table I).

Such conflicting data were difficult to interpret. Ganglionectomy or denervation usually stopped leg activity, but this was not invariably the case, and it was possible to produce activity in the amputated legs by injection of DDT. It was suspected that such results might be resolved in terms of DDT dose. Further experiments were then done with measured doses of DDT.

Localization experiments with controlled doses of DDT

It was immediately found that the effectiveness of ganglionectomy in abolishing motor effects was inversely related to dose (Table IV). That is, as the dose of DDT was increased ganglionectomy stopped movement in progressively fewer cases.

TABLE IV
Effect of ganglionectomy on symptoms after various doses of DDT

No. experiments	DDT	Results of ganglionectomy	
		Number resulting in complete cessation of activity	Number resulting in a reduction of activity
10	Usual moderate contact dose (5-10 mins. in DDT coated tube)	70%	30%
5	Excessive contact dose (approximately 2 hours in DDT coated tube)	20%	80%
25	5-30 mg. DDT per kg. injected intra-abdominally in emulsion**	68%	32%
12	60-70 mg. DDT per kg. injected intra-abdominally in emulsion**	50%	50%
15	130 mg. DDT per kg. injected intra-abdominally in emulsion**	7%	93%

* LD-50 for DDT injected intra-abdominally in emulsion is 20 mg. per kg. (Tobias, Kollros, and Savit, 1946a).

** Emulsion—1 per cent DDT, 1 per cent lecithin, 10 per cent peanut oil, 88 per cent of 0.9 per cent NaCl.

In all cases, however, even when movements were not entirely stopped they were both qualitatively and quantitatively changed. The high frequency tremulousness was always markedly reduced or entirely abolished and the slower movements were much diminished.

Nicotine, in low concentrations, is known to block synaptic transmission centrally as well as peripherally (Libet and Gerard, 1938; Pringle, 1939) but not axonal transmission. When applied to the cockroach ganglion there is an initial burst of electrical hyperactivity (100-800 impulses per sec.) followed by electrical silence (Pringle, 1939). As would be expected, such application of nicotine to a ganglion also produces great motor hyperactivity in the attached leg which can be abolished by amputating the leg (Yeager and Munson, 1945).

Now then, if nicotine applied to a ganglion in a concentration which did not

affect peripheral nerve were to stop DDT symptoms, this would be added evidence for the importance of the ganglionic cell bodies or synapses in the development and maintenance of such symptoms. After poisoned roaches became hyperactive the heart was excised. This did not decrease activity, but served to greatly diminish circulatory transport of solutions applied for local effects. Solutions were then applied as small droplets to the ganglion or a region of leg nerve exposed by cuticle excision.

Dilute nicotine solutions (0.01 per cent in insect Ringer) applied to the leg nerves of the normal or poisoned roach did not paralyze the leg. Typical DDT induced activity could not be stopped in this fashion. This was almost surely not due to failure of nicotine to reach the nerve since spontaneous movement as well as that following electrical stimulation of the ganglion could be stopped by a similar

TABLE V

Effect of locally applied nicotine and novocaine on motor symptoms of DDT poisoning after various doses of DDT*

No. experiments	DDT**	Number of experiments in which activity was modified			
		1.0% novocaine	0.01% nicotine		
		Injected into tibia	Injected into tibia	Applied to ganglion	
		Complete inactivity	Complete inactivity or reduced activity	Activity stopped	Activity reduced
4	10-30 mg. per kg. applied to body surface 18 hours before	100%	0%	100%	0%
9	100 mg. per kg. applied to body surface 18 hours before	100%	0%	77%	22%
13	500 mg. per kg. applied to body surface			23%	77%
4	1000 mg. per kg. applied to body surface			0%	100%

* All experiments on cardiectomized roaches to prevent circulatory removal of substances applied for local effect.

** DDT applied to surface in acetone. LD-50 for DDT so applied is 10 mg. per kg. (Tobias, Kollros, and Savit, 1946a).

administration of 1 per cent novocaine. When, however, this nicotine solution was applied to a ganglion (in the same normal or poisoned animal in which it was ineffective on peripheral nerve) there was a short-lived burst of great activity in the legs of the segment, followed by complete immobility or markedly decreased activity. Since the nicotine was effective in concentrations which did not block peripheral nerve, it was concluded that it was acting by blocking ganglionic synapses and not by spill-over to the emerging nerve roots. It is clear (Table V) that, as in the case of ganglionectomy, the immobilizing effect of nicotine decreased as the dose of DDT increased, and, as was also true after ganglionectomy, if nicotine did not stop activity it considerably decreased and modified it.

DISCUSSION

It is clear that DDT can produce motor symptoms by effects peripheral to the ganglion. It is equally clear, however, that the ganglion plays a role in the initiation and maintenance of symptoms and that this role is to some extent dependent upon DDT dose.

Roeder and Weiant (1946) found that, in the cockroach, very low concentrations of DDT can initiate centripetally directed, high frequency (300–400 per sec.), temporally irregular bursts of nerve impulses, presumably excited by action of DDT on the campaniform sensilla (presumptive proprioceptors). There was no evidence of any muscle movement which might have initiated such centrally directed impulses. Welsh (1946) has demonstrated that DDT in very low concentrations can also favor repetitive response of motor fibers (*Cancer irroratus*) to a stimulus normally evoking single responses, and Yeager and Munson (1945) have concluded that high concentrations can produce similar changes in the cockroach.

The results of ganglionectomy, in the cockroach (surgical or nicotine inactivated), after various doses of DDT, support the view that the initiation and continuation of the hypermotor symptoms of DDT poisoning after low doses of DDT require an intact sensori-motor reflex arc, and that random afferent impulses in sensory nerves may indeed, as suggested by Roeder and Weiant, excite motor neurones in the ganglion to initiate incoordinated muscular activity. From the experiments here reported, this would appear to be a part of the common sequence of changes in the roach poisoned by uncontrolled contact doses. The fact that ganglionectomy becomes less and less effective as the dose of DDT is progressively increased would suggest that larger amounts of DDT may act directly on motor nerves. Obviously, these data do not rule out a possible direct action on muscle. Within the dose ranges which have been used there is, however, no conclusive evidence for a direct action on muscle. This is not to say that such action could not occur at some sufficient dosage level. In addition, ganglionectomy is seen to have stopped the rapid tremulousness after any dose which was tried, suggesting that the high frequency movements may be initiated reflexly rather than by direct action on motor fibers at large as well as at low doses of DDT. This general picture is compatible with the subsidence of high frequency tremulousness before subsidence of slower muscular activity. If the former is reflex and the latter due to direct nerve action one might expect this order of dropping out on the basis of much greater fatigability for the reflex arc than for the nerve trunk.

Pattern development of symptoms

It has been claimed (Laüger, Martin, and Müller, 1944) that if DDT be put on one leg of a fly the development and progression of symptoms follow a definite, orderly and reproducible path from leg to leg. Such a phenomenon might be very important indeed for an understanding of the mechanism of DDT action. The authors have not been able to confirm this finding.

CONCLUSIONS

1. Neither decapitation, section of one or several nerve cord connectives nor complete transection of the entire insect body at one or several levels between nerve cord ganglia prevents the development of the typical motor effects of DDT in any

of the legs of the cockroach. After combined antero-posterior isolation of a nerve cord ganglion, even median sagittal section of the ganglion does not prevent motor symptoms in the legs still attached to the lateral ganglionic cell masses. Therefore, the anatomical elements necessary for development of the motor symptoms of DDT are contained within the lateral half of a body segment which contains the lateral half of a ganglion, leg nerves and peripheral structures.

2. Since the motor symptoms of DDT poisoning can occur in amputated legs, in legs whose nerves have been cut, and in legs whose segmental ganglia have been destroyed, it is possible for DDT to produce its motor effects by action on some structure or structures peripheral to the segmental ganglion.

3. The motor symptoms of DDT poisoning can be stopped or diminished in a leg by ganglionectomy, leg nerve section, or ganglion synaptic block with nicotine. The effectiveness of these procedures is in inverse relation to the dose of DDT administered. These findings suggest that, in the cockroach, low doses of DDT may excite motor fibers reflexly by impulses fired into the ganglion over afferent nerve fibers, whereas high doses may act on elements on the motor side of the ganglion and thus not require an intact reflex arc. Since ganglionectomy stops the fast component of the hypermotor activity, however, equally well after large or small doses of DDT, this component may be reflexly initiated and maintained after all doses of DDT.

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TILLINA MAGNA: MICRONUCLEAR NUMBER, ENCYSTMENT AND VITALITY IN DIVERSE CLONES; CAPABILITIES OF AMICRONUCLEATE RACES

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It is well established that the number of micronuclei in *Tillina magna* is highly variable. For example, Gregory (1909) found 6-10, and Ilowaisky (1921), in a ciliate which he called *Pseudocolpoda cochlearis cienkowski*, reported 2-6. An examination of Ilowaisky's text and figures shows conclusively that his ciliate was *T. magna* Gruber (1879) as Kahl (1931, p. 282) pointed out. Kahl apparently regarded six as the typical number. Bresslau (1922) observed the nuclei in sufficient detail to note the extrusion of macronuclear material at division and the presence of several micronuclei, though he reported no counts of their actual number. The writer (1946), in a study dealing chiefly with the history of the nuclei during division and encystment, counted the number of micronuclei in 100 individuals (50 active and 50 encysted) of each of three clones, and found that it varied from 6 to 11 in one clone and from 4 to 6 in the other two. Thus the number was found to vary in different individuals of the same clone, and the mean number was found to vary in different clones. Active specimens and resting cysts of any particular clone had on the average like numbers of micronuclei. Contrary to statements in the literature, it was shown that the micronuclei divide at the time of cell division, and not indiscriminately or without regard to cell division. The mechanism by which two daughter cells may receive unlike numbers of micronuclei at division, thus accounting for variations in number within a clone, was described.

The significance of the wide variation in micronuclear number is unexplained. Structurally and physiologically an individual having only 4 micronuclei does not appear to be fundamentally unlike one having 11 micronuclei. The same condition prevails in the closely related species, *T. canalifera*, which I was formerly disposed to regard (1945) as identical with *T. magna*. However, on the basis of information furnished me by Dr. George W. Kidder, of Amherst College, it appears that *T. canalifera* merits recognition as a valid species, chiefly because of the very conspicuous nature of its canal system. In *T. canalifera*, Turner (1937) reported 4-14 micronuclei, though Burt, Kidder, and Claff (1941), in specimens obtained from the late Dr. Turner, found only one. Hence, it is clear that the micronuclear number may vary from 1 to 14, yet the evidence indicates that the uninucleate and multi-nucleate races were equally cultivable, vigorous, and capable of producing normal resting cysts.

The present study of *T. magna* was undertaken in order to obtain additional information concerning two points: (1) the normal variation in micronuclear number in various natural races and (2) the significance of such variation. The investigation of the first point is readily feasible, in that the micronuclei may be counted with absolute certainty in Feulgen preparations of favorably oriented resting cysts or

medium-sized trophic specimens. The investigation of the second point, though less suited to direct approach, is not impracticable. A number of questions arise, some of which submit to experimental analysis. For example, is the number of micronuclei related in any way to size, whether of trophic specimens, division cysts or resting cysts; to division rate; to vitality, meaning capacity to endure with undiminished vigor as generations pass; to ability to produce resting cysts; to the viability of such cysts; or to ability to excyst? Of the foregoing measurable characters, the following were selected as being most readily amenable to experimental investigation: ability to produce resting cysts, size and viability of such cysts, capacity to excyst, division rate and vitality. These then, will be considered in relation to micronuclear number, though not all of them will receive equal consideration. The study assumed unlooked-for interest when it became evident that three of the races were amiconucleate. Thus a comparison of the potentialities of micronucleate and amiconucleate clones became possible.

MATERIALS AND METHODS

Twenty clones of *T. magna*, to be designated numerically, were used in the study. The progenitors of these respective clones were collected in a meadow, known locally as Sparrow's Pasture, in the vicinity of Chapel Hill, North Carolina. Comparisons with clones from other sources were desirable, but unfortunately attempts to collect *Tillina* elsewhere in the Chapel Hill region, and in the vicinity of Stanford University, California, and Woods Hole, Massachusetts, were unavailing. In this study a clone refers to all the progeny which were derived asexually from a single resting cyst or trophic specimen. The intervention of encystment and subsequent excystment is not considered to be a valid reason for changing the clonal designation, since there is no evidence that encystment in *Tillina* involves a sexual process which might change the genetic constitution of the clone. (It should perhaps be recalled that *Tillina*, like its near relative *Colpoda*, reproduces within a thin-walled temporary cyst, from which usually four progeny emerge shortly as a result of two successive divisions. The term encystment, as used in this study, does not refer to these temporary division cysts, but to the protective or resting cysts.) It is not definitely established that all of the twenty clones were genetically different, since their histories prior to their period of laboratory life were unknown.

The progenitors of clones 1, 2, 6, 8, 9, 11, 12, 13, 15, 17, 18, and 19 were taken as active specimens on Sept. 10, 1945, and these clones were therefore cultured simultaneously in the early part of the study. Eight of the foregoing clones, namely, 6, 11, 13, 18, 15, 19, 1, and 17, have already been reported on briefly under the numerical designations 1 to 8, respectively (Beers, 1946a). In the present paper my original numerical designations of all clones have been changed for the convenience of the reader in using the accompanying tables. The progenitors of clones 3, 14, 16, and 20 were isolated on Feb. 4, 1946, when dried leaves and debris, after 8 months of storage at 19° C., were immersed in weak hay infusion; these clones were therefore cultured simultaneously. It is evident that they were derived from dried cysts. The progenitors of clones 4, 5, 7, and 10 were isolated on April 8, 1946, when moist leaves and debris, which had recently washed against the bases of willow saplings in the meadow, were immersed in hay infusion; these clones were

maintained in culture simultaneously toward the end of the study. They were undoubtedly derived from wet cysts.

An attempt was made to maintain each of the clones in pure-line culture for a period of 60 days. Sixteen of the clones were readily cultivable and continued with undiminished vigor throughout the period; four were intractable in that their division rates declined and the lines encysted well before the end of the period. Thus the laboratory histories of the clones varied, although the conditions of culture were uniform. The details, in relation to micronuclear number, will follow.

Each clone was cultured in depression slides in the form of four sub-lines. These were maintained at 23° C. in 0.05 per cent lettuce infusion to which suitable quantities of *Pseudomonas fluorescens*, grown on nutrient agar, were added as food. Previous experience has shown that this general procedure, combined with daily isolations and transfers to fresh environments, meets adequately the cultural needs of *Tillina* (Beers, 1944, 1945). Records were made daily of fission rates and other points of interest.

Surplus animals from the lines were stained on cover glasses by the Feulgen method in order to make micronuclear counts of active specimens. Small stock cultures of each clone furnished precystic specimens when the food supply neared depletion. These specimens were removed and allowed to encyst on cover glasses in the manner described by Beers (1946). Thus convenient preparations were available, first for making measurements of living cysts, and then for Feulgen staining. All measurements and micronuclear counts of cysts were made on *single* resting cysts. These are the common type. They are practically spherical and therefore well suited for making accurate measurements.

NORMAL VARIATION IN NUMBER OF MICRONUCLEI

The data bearing on diversity in micronuclear number in the twenty clones are summarized in Table I, in which the clones are arranged and numbered in the order of decreasing mean numbers of micronuclei. The data, ignoring for the present the mean diameters of resting cysts, are largely self-explanatory. A few points deserve special mention.

In any particular clone both active specimens and resting cysts showed practically the same extremes of variation (range) in micronuclear number and had essentially the same mean number of micronuclei.

In different clones the mean numbers of micronuclei were extremely variable. Some clones (e.g., 1 and 2) had consistently high mean numbers; others (e.g., 16 and 17), consistently low numbers, with many intergrades between these extremes.

Clones 18, 19, and 20 were amiconucleate. This statement is not based on casual observation, but on an intensive study of these clones. In trophic specimens and resting cysts the micronuclei of *Tillina* are not disposed toward secretiveness. They are never imbedded in the macronucleus. Each has an endosome which stains intensely and conspicuously by the Feulgen method. In mature resting cysts only the rod-shaped or ellipsoid macronucleus and the micronuclei stain to any appreciable extent; there is nothing in the cytoplasm to conceal the micronuclei. In trophic specimens it is true that the food vacuoles also stain, but the micronuclei always lie in the clear peri-macronuclear space and are not in a position to be concealed by the vacuoles. Moreover, considerable numbers of individuals of clones

18, 19, and 20 were stained. These included not only the usual resting cysts and medium-sized trophic specimens, but also young cysts, cysts in the process of excystment, and individuals just excysted. None showed a micronucleus, whereas individuals of the remaining seventeen clones, stained at the same time by the same method, invariably showed micronuclei.

The individuals of some clones (e.g., 1, 3, 5, 12) showed great diversity in micronuclear number within the clone. This fact is brought out clearly by the range which is cited for these clones, and it is further emphasized by the high standard deviations in the clones. Clone 12 showed the greatest degree of heterogeneity in that the range in micronuclear number extended from 2 to 11, with all intervening numbers being represented. On the other hand, some clones (e.g., 2, 4, 8; 13, 15, 16, 17) were relatively homogeneous, with narrow ranges and low standard deviations. Other clones lay between these extremes. Only the amicro-nucleate clones showed complete homogeneity.

Thus, it is seen that individuals of a clone exhibit varying numbers of micronuclei, that clones differ with respect to their mean number, and that amicro-nucleate clones exist in nature.

The mean number of micronuclei in the 850 micronucleate active specimens of Table I (representing 17 clones) was 7.06; the mean number in the 850 micro-nucleate cysts was 7.08. Unfortunately, the number in the progenitor of each clone

TABLE I

Tillina magna. Variation in number of micronuclei in twenty clones; relation of micronuclear number to size of cysts. The clones are numbered and arranged as the mean number of micronuclei (average of means for fifty active specimens and fifty resting cysts) decreases.

Numerical designation of clone	Range in number of micronuclei		Mean number of micronuclei \pm standard deviation		Mean diameter of 50 resting cysts in microns \pm standard deviation
	50 active specimens	50 resting cysts	50 active specimens	50 resting cysts	
1	10-16	9-16	12.90 \pm 1.87	12.32 \pm 1.93	85.76 \pm 9.65
2	8-12	9-12	10.48 \pm 0.94	10.96 \pm 0.87	82.94 \pm 7.65
3	7-14	6-13	9.62 \pm 2.30	9.24 \pm 2.08	79.68 \pm 8.72
4	7-10	7-10	8.52 \pm 0.82	8.60 \pm 0.95	88.62 \pm 7.25
5	6-14	6-12	8.17 \pm 1.95	8.28 \pm 1.66	88.36 \pm 9.61
6	6-10	6-10	7.56 \pm 1.28	7.78 \pm 1.24	93.60 \pm 6.21
7	5-10	5-10	7.25 \pm 1.28	7.38 \pm 1.36	92.44 \pm 7.08
8	6- 9	6- 9	7.16 \pm 0.85	7.06 \pm 0.92	78.50 \pm 7.82
9	5- 9	4- 9	6.52 \pm 1.15	6.74 \pm 1.21	84.16 \pm 8.62
10	4- 8	4- 8	5.98 \pm 1.52	5.94 \pm 1.46	85.42 \pm 5.28
11	5- 8	5- 9	5.90 \pm 1.03	5.76 \pm 0.96	86.14 \pm 5.34
12	2-10	2-11	5.74 \pm 2.41	5.90 \pm 1.97	85.64 \pm 5.32
13	4- 6	4- 6	5.12 \pm 0.42	5.10 \pm 0.45	80.64 \pm 8.92
14	4- 8	4- 8	5.12 \pm 1.51	4.98 \pm 1.42	84.18 \pm 4.76
15	4- 6	4- 6	4.86 \pm 0.53	5.20 \pm 0.57	81.60 \pm 8.41
16	4- 6	4- 6	4.92 \pm 0.63	4.98 \pm 0.75	80.92 \pm 7.37
17	3- 5	3- 5	4.28 \pm 0.75	4.14 \pm 0.74	91.42 \pm 6.87
18	—	—	0	0	86.28 \pm 5.63
19	—	—	0	0	84.32 \pm 10.36
20	—	—	0	0	81.52 \pm 5.34

is unknown, since the micronuclei cannot be identified in living specimens. It seems reasonable to assume that the progenitor of each had a number approximately equivalent to the mean which was determined for the clone, and that it produced some offspring having fewer, and some having more, than its own number.

It is well known that the number of micronuclei is variable in many species of ciliates. Thus, *Paramecium multimicronucleatum* has 2 to 7 (Powers and Mitchell, 1910), though usually 4 (Wenrich, 1928); *Spathidium spathula*, 6 to 9 (Maupas, 1888, p. 247); *Urostyla grandis*, 10 to more than 40 (Tittler, 1935); and *Stentor coeruleus*, 10 to 42 within a single clone (Schwartz, 1935). On the whole, such variations within a species appear to have little effect on the structure or behavior of the individuals and to be without functional significance. This general conclusion is supported by the observations on *T. magna* which follow immediately.

NUMBER OF MICRONUCLEI IN RELATION TO VARIOUS ASPECTS OF CYSTMENT

All the clones produced normal resting cysts upon the depletion of the food supply in small stock cultures prepared with surplus animals from the lines. Furthermore, all the specimens in such cultures encysted; none persisted in prolonged swimming, thereby to perish of starvation. Hence, it is clear that the ability to encyst is not dependent on the presence of the micronucleus, since amiconucleate as well as micronucleate clones were able to encyst. Moreover, the cysts of all the clones remained viable for many months. They could be activated at any time after the fourth day by immersion in distilled water or 0.05 per cent lettuce infusion. From 2 to 2.5 hours were required for emergence at 23° C., and practically 100 per cent of the specimens excysted. No precise figures are given here, since the percentage of excystment under various conditions has been dealt with in a previous paper (Beers, 1945) and the present study contributes nothing new on this point. However, the present results show clearly that the viability of resting cysts and their capacity to excyst are in no wise related to the presence of a micronucleus, or to the number of micronuclei. Well over 90 per cent of the cysts produced in the various clones were single ones; double cysts appeared sporadically, some in amiconucleate clones, some in micronucleate. Amiconucleate cysts undergo the usual colpodid type of macronuclear reorganization, involving the extrusion of a portion of the macronuclear substance into the cytoplasm (Taylor and Garnjobst, 1941; Burt, Kidder and Claff, 1941; Beers, 1946).

The size of the cysts in different clones was made the subject of special study, for it was thought that the number of micronuclei might affect the size of the cysts. The diameters of fifty living single cysts of each clone were measured, each measurement extending from the outer surface of the ectocyst of one side to the corresponding surface of the other. The results of these measurements are included in Table I. An inspection of the table shows at once that the calculation of coefficients of correlation between micronuclear number and cyst size would be of little value, since cyst size is independent of micronuclear number. For example, if we consider certain extremes in micronuclear number, it is seen that the cysts of clone 1 had a mean diameter of about 85 μ , and those of clone 19 a diameter of 84 μ , with approximately equivalent standard deviations. Clones 2 and 20 and clones 4 and 18 constitute other examples of extreme disparity in micronuclear number with close agreement in cyst size. Among the micronucleate clones, other examples of

wide divergence in micronuclear number, yet with general uniformity in cyst size, are furnished by clones 3 and 16 and by clones 6 and 17. On the other hand, some clones having widely divergent micronuclear numbers produced cysts of dissimilar sizes, e.g., clones 3 and 17 and clones 6 and 13. Thus it is seen that clones having widely different micronuclear numbers may produce cysts of equivalent mean sizes or of dissimilar mean sizes. It must be concluded that there is no relation between the number of micronuclei and the size of the cysts.

The same conclusion is reached if we adopt another approach and consider cyst size in clones which had similar, or only slightly different, micronuclear numbers. For example, clones 6 and 7 had similar mean numbers of micronuclei and they produced cysts of equivalent mean sizes; clones 15 and 16 constitute a second example. On the other hand, clones 7 and 8 had similar micronuclear numbers, but they produced cysts which differed significantly in mean size; clones 16 and 17 furnish another example. Hence, clones having similar mean micronuclear numbers may produce cysts of equivalent mean sizes or of different mean sizes. Again, it is evident that there is no relation between number of micronuclei and size of cysts. The mean diameter of the 850 micronucleate cysts of Table I was $85.23\ \mu$; that of the 150 amiconucleate cysts was $84.04\ \mu$.

A word concerning the size of individual cysts may be of interest. In any particular clone of *T. magna*, whether micronucleate or amiconucleate, there is usually wide variation in cyst size, even though the cysts under immediate consideration are all produced in the same small stock culture—meaning a Columbia culture dish containing 1 cc. of fluid. Cysts in such a culture often vary in size from $75\ \mu$ to $95\ \mu$; extremes of $64\ \mu$ and $104\ \mu$ have been noted. The factors which affect cyst size appear to be of a complex physiological nature and are therefore not readily identifiable.

The point of greatest interest in this consideration of various aspects of cystment is the fact that amiconucleate and micronucleate clones behaved alike; clearly the micronucleus of *T. magna* plays a negligible role, if any, with reference to encystment, viability of cysts, excystment, macronuclear reorganization within the cysts, and cyst size.

NUMBER OF MICRONUCLEI IN RELATION TO DIVISION RATE AND VITALITY

The cultural histories of the twenty clones are presented in Table II. Clones 1, 2, 3, and 5 could not be maintained in culture for the arbitrary period of 60 days. The remaining clones were maintained with undiminished vigor and were discontinued at the end of the period.

First, the division rates of the sixteen vigorous clones will be considered in relation to micronuclear number. Clones 4, 6, 7, and 8, as has been seen, had relatively high micronuclear numbers (means, about 7 to 8.5). The total average number of generations produced by the four sub-lines of these respective clones varied from 149 to 174. Thus the clones themselves varied with respect to mean division rate. Clones 9–12 had intermediate micronuclear numbers (means, 6 to 6.5); these clones produced from 149 to 167 generations. Clones 13–17 had low micronuclear numbers (means, 4 to 5); they produced from 160 to 176 generations. Thus clones having intermediate or low micronuclear numbers produced in general as many generations, and had therefore the same division rates, as clones having relatively high micronuclear numbers.

Amicronucleate clones 18–20 produced from 154 to 172 generations. Thus the amicronucleate clones had approximately the same division rates as the micronucleate clones; e.g., clone 19 produced almost as many generations as clone 4; clone 18 produced more than clone 10; clone 20 produced about as many as clone 9 or 13. Many kinds of comparisons are possible, and the reader may choose to make other comparisons between amicronucleate and micronucleate clones. For example, clones 18–20 had higher division rates (i.e., produced more generations) than clones 8, 10, and 14; but clones 18–20 had lower division rates than clones 4, 7, and 16. Thus the general conclusion that amicronucleate clones have the same division rates as micronucleate clones is not invalidated. Sections of amicronucleate division cysts showed that the macronucleus undergoes the usual reorganization after each of its divisions, as in normal micronucleate cysts (Burt, Kidder and Claff, 1941; Beers, 1946).

Next, the vitality of the sixteen vigorous clones must be considered. An examination of the number of divisions produced in the successive 5-day periods in any particular clone shows that the clone was dividing as rapidly at the end of the experiment as at the beginning. Within the time limits of the experiment, the clones showed no decrease in vitality as measured by the division rate. How long the sixteen clones would have continued without diminution in vitality is a question that cannot be answered on the basis of the available data. The important findings are these: Some clones which have relatively high micronuclear numbers are as vigorous as those which have low numbers; amicronucleate clones are fully as vigorous as many micronucleate clones.

Clones 1, 2, 3, and 5 must receive special consideration. As has been said, these clones could not be maintained in culture for the duration of the 60-day experimental period. Clone 1 showed a rapid decrease in fission rate and encysted on the fourth day. Some of the cysts were activated and new lines were established. These in turn declined shortly and encysted. Three additional attempts were made to culture clone 1; each time the lines encysted after 3–5 days. Indeed, clone 1 was so refractory that without these repetitions it would have been impossible to obtain sufficient specimens for the usual number of micronuclear counts. Clones 2, 3, and 5 likewise could not be maintained in culture for 60 days. Their histories are presented in sufficient detail in Table II. Following the encystment of the original lines of these clones, new lines were established with excysted specimens, but they also declined and encysted after 3–4 weeks of culture. It may be maintained that the decline and encystment of these four clones resulted from a failure to meet their cultural needs. However, the conditions of culture were adequate for a total of sixteen clones, and it seems not unreasonable to assume that they were likewise adequate for clones 1, 2, 3, and 5 and to conclude that these clones declined as a result of intrinsic factors.

It has been shown that clones 1, 2, and 3 had higher micronuclear numbers than any of the other clones. Clone 5 likewise had a high number, though slightly lower than clone 4, which was cultivable. The evidence indicates that a large number of micronuclei may be detrimental to the welfare of the organism and incompatible with high vitality, but the number of such clones studied was too small to justify a general conclusion. On the whole, the results show that in *T. magna* the rate of division and the vitality of the race are in no wise related to the number of micro-

TABLE II

Tyllina magna. Relation of number of micronuclei to division rate and vitality in twenty clones maintained in *p-re*-line culture, usually for sixty days. Four clones having high micronuclear numbers encysted before the expiration of sixty days; the remaining sixteen continued with undiminished vigor, though their mean micronuclear numbers (see Table I) varied from 8.56 to 0.

Successive five-day periods	Total average number of divisions per line for successive periods																			
	Cl. 1	Cl. 2	Cl. 3	Cl. 4	Cl. 5	Cl. 6	Cl. 7	Cl. 8	Cl. 9	Cl. 10	Cl. 11	Cl. 12	Cl. 13	Cl. 14	Cl. 15	Cl. 16	Cl. 17	Cl. 18	Cl. 19	Cl. 20
1	5.5*	13.5	11.5	14.5	15.5	12.5	16.0	11.5	12.5	11.5	13.0	13.0	14.5	14.5	14.5	14.0	12.5	11.5	12.5	13.5
2		11.0	9.5	16.5	12.5	13.0	15.0	12.5	11.5	10.5	14.0	14.5	15.0	13.0	12.5	14.0	11.5	12.5	13.5	12.5
3		9.5	12.5	13.5	11.5	14.5	12.5	12.5	13.0	12.5	12.5	15.5	13.5	12.5	13.0	15.0	12.5	16.5	14.5	11.5
4		7.5*	10.0	14.0	10.0	11.5	14.0	13.0	12.0	15.5	11.5	13.5	14.5	11.5	16.5	15.5	13.5	12.5	15.0	14.5
5			8.5	13.0	7.0	15.0	12.5	11.5	14.5	14.5	14.0	12.5	15.0	14.5	14.0	14.5	14.5	11.0	15.5	12.5
6			9.5	16.5	0.5*	12.5	14.5	11.5	15.0	13.5	13.5	13.5	12.5	12.5	15.5	16.5	13.5	13.0	16.5	14.5
7			7.8	15.0		14.0	15.0	12.5	15.5	12.5	12.5	14.5	14.5	13.5	13.5	14.5	14.0	13.5	14.5	15.5
8			2.2*	13.5		13.5	16.5	13.5	14.0	11.5	14.5	15.0	13.5	14.5	12.5	13.0	15.0	14.5	13.0	14.5
9				14.5		13.0	13.5	11.5	16.0	12.5	13.0	13.5	11.5	11.5	14.5	14.5	14.5	12.5	14.5	13.5
10				14.0		16.0	14.0	12.5	13.5	10.5	15.0	12.5	12.5	14.0	16.0	16.5	13.5	11.0	13.5	12.5
11				15.5		12.5	15.5	13.0	12.5	13.5	12.5	14.5	14.5	12.5	13.5	14.5	13.0	12.5	15.0	13.5
12				14.0		14.0	14.5	13.5	12.0	10.5	12.5	15.0	15.0	13.0	14.5	13.5	12.5	13.0	14.0	15.5
Total number of generations	5.5	41.5	71.5	174.5	57.0	162.0	173.5	149.0	162.0	149.0	158.5	167.5	166.5	157.5	170.5	176.0	160.5	154.0	172.0	161.0

*Cl. 1. Encysted after 4 days.

*Cl. 2. Encysted after 20 days.

*Cl. 3. Encysted after 37 days.

*Cl. 5. Encysted after 26 days.

nuclei, or even to the presence of a micronucleus, since amiconucleate clones proved to be as vigorous as micronucleate clones.

The origin of the amiconucleate races cannot be accounted for with certainty. It is agreed that such races may arise at any of four periods in the life of ciliates generally: at endomixis, by the transformation of all the reconstruction micronuclei into macronuclei; at autogamy or conjugation, by an analogous transformation of all the derivatives of the synkaryon or amphinucleus into macronuclei; or at division, by an unequal distribution of the micronuclei to the daughter cells. By studying dividing individuals of an unusual race of *Paramecium caudatum*, Wichterman (1946) observed the simultaneous production of bimicronucleate and amiconucleate daughters. In *T. magna* conjugation is of rare occurrence, and endomixis and autogamy are unknown. Therefore, it is likely that amiconucleate races usually take their origin in an unequal distribution of the products of division. However, it should not be concluded from the present results that 15 per cent of all *Tillina* clones are amiconucleate; actually such clones appear to be very exceptional. I have stained many specimens during a period of 8 years; all these specimens had micronuclei, except for the members of clones 18-20.

DISCUSSION

The functional significance of the nuclear dimorphism of the Euciliata has long held the attention of protozoologists. In general, the dimorphic condition has been viewed as representing a segregation of idiochromatin and trophochromatin, the former in the micronucleus, the latter in the macronucleus. It was originally assumed, since ciliates normally possess both types of nuclei, that both are necessary for the continued survival of the individual. Although the precise functions of the respective nuclei are difficult to determine, two lines of investigation have supplied pertinent findings, namely, a study of the capabilities of amiconucleate ciliates of natural occurrence and a study of regenerative capacity, survival, and reproduction in ciliates which have been deprived experimentally of either nucleus, or of both, whether by merotomy or other operative procedures.

The existence of naturally occurring amiconucleate races has long been conceded by such authorities as Woodruff (1921), Calkins (1930), and Reichenow (1929, p. 29) and is now accepted as a fact. The potentialities of these races, as revealed by intensive laboratory study, have demonstrated that the micronucleus is not at all necessary for the maintenance of the essential vital processes of the individual, whereas the macronucleus is indispensable. Aside from the absence of a micronucleus and the manifest inability to carry to completion such processes as endomixis, autogamy and conjugation, amiconucleate races of many ciliates do not differ structurally or physiologically from micronucleate ones.

Thus, Dawson (1919; 1920) maintained an amiconucleate race of *Oxytricha hymenostoma* in pure-line culture for 289 generations (4 months) and in small mass cultures for 5 months longer. The absence of micronuclei did not prevent the animals from attempting to conjugate, but these attempts were abortive. Woodruff (1921) cultured amiconucleate races of *Oxytricha fallax* and *Urostyla grandis* for 246 and 128 generations, respectively, and maintained a race of *Paramecium caudatum* in pure-line culture long enough to determine that it was definitely amicro-

nucleate. A few pairs of conjugants occurred in mass cultures of *O. fallax*, but they failed to live when isolated.

Amicronucleate races of other ciliates have likewise been isolated and cultured long enough to demonstrate not only their viability but their sustained vigor and good health. Among these are the following: (1) *Spathidium spathula*. Moody (1912) was unable to find micronuclei in her specimens, though she was able to culture them for 218 generations. It is evident that they were amicronucleate, since the micronuclei of *Spathidium* were observed and counted by Maupas and were found regularly by Woodruff and Spencer (1922). (2) *Didinium nasutum*. Patten (1921) cultured an amicronucleate race, which was derived from an exconjugant of a normal micronucleate race, for 652 generations. Conjugation occurred in the amicronucleate race, but the exconjugants invariably died. The resting cysts were likewise inviable. It is evident that the race arose through the faulty reorganization of the exconjugant. (3) *Paramecium bursaria*. Woodruff (1931) cultured a race characterized by micronuclear instability for 7 years. Neither endomixis nor conjugation was observed. The race was originally bimicronucleate; later it was variable, exhibiting from 1 to 4 micronuclei; then for about 4 years it was unimicronucleate; finally, a derived race showed no micronucleus, although this race was apparently as healthy and vigorous as its bimicronucleate ancestors which were isolated 7 years earlier. Woodruff (p. 543) aptly points out that "whatever function the micronuclear apparatus plays, the somatic life of the animals is not obviously influenced by profound variations in volume or in distribution of micronuclear material." (4) *Urostyla grandis*. Tittler (1935) found amicronucleate individuals in stock cultures which previously contained only micronucleate specimens. They were indistinguishable externally from their micronucleate progenitors, and they flourished in mass cultures for 2 years. Their macronuclear divisions followed the usual complex pattern characteristic of the species. The race produced resting cysts, some of which could be excysted, although endomixis, which usually occurs in the precystic forms, was absent. Evidently some of the cysts were not entirely normal, since they showed a tendency to disintegrate, perhaps because of the omission of endomixis. (5) *Colpoda steini*. Piekarski (1939) studied comparatively the structure and reproduction of a micronucleate and an amicronucleate race and was able to culture the latter for approximately 6 years. Both races were equally cultivable and vigorous. They reproduced within division cysts from which four progeny regularly emerged and they produced normal resting cysts. They showed the same sequence of events in the division of the macronucleus. These events are of special interest, in that eight chromatic (Feulgen-positive) bodies appear in the macronucleus of the young division cyst. Ultimately each of the daughters receives two of them, and thus the behavior of these bodies suggests an equational distribution of chromosomes. Piekarski concludes that the absence of a micronucleus had no recognizable effect on the activities of the animals.

The present study of amicronucleate races of *Tillina magna* likewise demonstrates the adequacy of the macronucleus, not only for long-continued reproduction accompanied by sustained vigor, but also for encystment and excystment. Thus endowed with the ability to produce viable resting cysts, these races would seem to be capable of indefinite survival, even under the changeable conditions of a natural environment.

Still more remarkable, in connection with the capabilities of amiconucleate races, are the observations of Sonneborn (1940) and Kimball (1941) on mating types. In certain races of *Paramecium aurelia* Sonneborn found a small percentage of animals which, upon undergoing autogamy or conjugation, developed a new macronucleus from a fragment of the old macronucleus. Since the micronuclei commonly disappeared in clones produced by these animals and since the mating type never changed at macronuclear reorganization, Sonneborn concludes that "hereditary characters (including mating type) of clones from macronuclear regenerates cannot be directly determined by micronuclei, for they persist in the absence of micronuclei. Mating type must be determined by the macronucleus. . . ." Kimball was able to assign amiconucleate specimens of *Euplotes patella* to definite mating types, since they paired readily with individuals of known mating type. He concludes that "the micronucleus is thus unnecessary for an animal to be of a definite mating type."

With reference to the role of the nuclei in the regeneration of ciliates following merotomy, the results obtained with different species are not always in complete accord. The physical properties of the cytoplasm constitute an experimental variable; a fluid cytoplasm or a rigid pellicle may interfere with the closure of an injury and thus affect regeneration adversely. Balamuth (1940) has presented an excellent review of the extensive literature on this subject. For the present only a few representative examples of regeneration in ciliates will be considered, with special reference to the nuclear components of the merozoa (cell fragments).

It is well known that enucleate fragments of ciliates can neither regenerate nor continue to live, whereas nucleate fragments regenerate successfully and pursue normal lives. These conclusions are particularly evident in Balamuth's six-page tabular summary of the findings on regeneration in the ciliates. As a rule the macronucleus and micronucleus cannot be separated, and a nucleate fragment, as in Dembowska's work (1925) on *Stylonychia mytilus*, usually means one having both macronuclear and micronuclear material.

However, some investigators have succeeded in obtaining nucleate merozoa of the two types desirable for an experimental analysis of the role of the individual nuclei in regeneration; namely, macronucleate (without micronuclei) and micronucleate (without any part of the macronucleus). Reynolds (1932), for example, in work on an amiconucleate *Oxytricha fallax*, found that various types of macronucleate merozoa could regenerate their missing parts and resume their normal physiological activities. Schwartz, using microdissection techniques, was able to remove the entire macronucleus from *Stentor* and yet leave a number of micronuclei in the specimens. These micronucleate individuals never survived. By means of successive operations, he was also able to remove all the micronuclei from a few specimens, leaving a portion of the beaded macronucleus in place. These macronucleate individuals regenerated and could be cultured as pure lines. Thus he produced experimentally an amiconucleate race, which as regards size and division rate was not different from the normal controls. Bishop (1943), employing the ultra-centrifuge as a means of obtaining merozoa of *Oxytricha fallax*, obtained sixty-seven macronucleate fragments, all of which regenerated, and seven micronucleate fragments, none of which regenerated. Twelve of the regenerated macronucleate individuals were cultured as amiconucleate pure lines. Bishop concluded (p. 451) that "the lack of micronuclear material makes no difference in the regenerative capacity, division rate, motility or morphology of *Oxytricha fallax*."

On the other hand, there is evidence that in some forms the micronucleus, as well as the macronucleus, is necessary for regeneration and survival. Thus Reynolds found that both nuclei are necessary for the regeneration of merozoa of *Euplotes patella*. This observation is in accord with the results of Taylor and Farber (1924), who, by means of a micro-pipette, removed the micronucleus from fifty specimens of *E. patella*, all of which died within a few days. None produced more than four progeny. Hence, these authors conclude that "the micronucleus plays more than a purely germinal role in the life history of *Euplotes patella*." However, the situation in *E. patella* is confused, for some of Kimball's unimicronucleate double animals produced viable amicronucleate individuals at division. Some of them survived as clones, though with a low division rate; one such amicronucleate clone survived for 341 days. The results show, according to Kimball (p. 30), "that the micronucleus is not essential for continued life in at least some clones of *Euplotes patella*, though its absence results in a marked decrease in vigor." In various species of *Uronychia* (Young, 1922), and in *Uroleptus mobilis* (Tittler, 1938), *Spathidium spathula* and *Blepharisma undulans* (Moore, 1924) both types of nuclei appear to be necessary for the complete regeneration, growth and division of merozoa.

Thus the evidence afforded by the long-continued culture of a number of naturally occurring amicronucleate races demonstrates conclusively that the macronucleus alone suffices for the maintenance of the vegetative life of the organism—meaning by vegetative life such diverse activities as locomotion, food capture, digestion, assimilation, growth, excretion, respiration, reproduction, and maintenance of cell proportions and form. On the basis of this evidence the normal role of the micronucleus in vegetative life appears to be one of relative passivity. The evidence from operative procedures shows that in many ciliates the macronucleus alone is adequate for complete regeneration, as well as for subsequent growth and division, whereas in other ciliates the micronucleus also is necessary. There is no authenticated case on record in which the micronucleus alone is adequate for the maintenance of vegetative functions or for regeneration. Balamuth's thorough survey of the literature leads him to make the following comment in his summary: "Of the dual nuclear apparatus, only the macronucleus can be shown to function in the actual regenerative process. The role of the micronucleus in this connection is as yet unclear; apparently it is more important in the viability of some forms than in others." On the whole, the evidence tends only to emphasize the importance of the macronucleus and to attest to the validity of Calkins' comment (1930, p. 161) on this organelle: "Far from being negligible it is on the contrary probably the most important element of the cell in matters of metabolism, reorganization, and continued cell life."

The tendency to underestimate the importance of the macronucleus in the life of the organism results probably from its apparent monotony of structure. Lacking chromosomes, its division is usually unspectacular. Nevertheless, its mode of origin is not an incidental phenomenon in the life of the ciliate, for both macro- and micronucleus almost invariably have a common and simultaneous origin. Usually they develop from the synkaryon of the conjugant, by divisions which appear to be equational. Again, they develop from the synkaryon of autogamy or from the endomictic micronucleus. Thus they inherit equally from a common nucleus of origin, and each receives equivalent chromatin elements, chief among which are presumably the genes. In the definitive micronucleus these elements retain the ability to or-

ganize periodically as chromosomes, and thereby to arrest the attention of the observer. Once in the definitive macronucleus, on the contrary, they never again assemble in the form of chromosomes. However, it is possible that they are distributed at macronuclear division by a mechanism which is fully as effective as the mitotic distribution of chromosomes, though less conspicuous. For example, it is not impossible that they are represented in multiplicate in the macronucleus and distributed at random throughout its substance. Thus each daughter at division would be reasonably assured of receiving representatives of every type of chromatin element. The behavior and the potencies of the macronuclear fragments of Sonneborn's unusual specimens of *Paramecium aurelia* indicate a multiplicate representation of the chromatin elements. In these specimens the forty or more macronuclear fragments grew and segregated during subsequent cell divisions, until there was only one in each cell. Thus each fragment was adequate for the regeneration of a complete macronucleus and for the continued life of the organism, even in the absence of micronuclei. Hence, Sonneborn concludes that "the normal macronucleus must contain at least forty complete and discrete genomes." A more precise mechanism for the distribution of the chromatin elements, involving, for example, a differential streaming of genetically equivalent elements toward opposite ends of a polarized macronucleus, may be postulated. Regardless of the type of mechanism involved in the distribution of the chromatin elements of the macronucleus at division, the fact remains that inheritance in an amiconucleate ciliate is no less precise, to judge by the structure and physiological activities of the offspring, than in a micronucleate ciliate. The fact that the behavior of the macronucleus does not conform to the chromosome theory of heredity in *sensu stricto*, in that chromosomes are absent, may mean simply that a different mechanism for the distribution of the genes is involved.

Whether the macronucleus of amiconucleate ciliates may justifiably be regarded as an amphinucleus—one containing idiochromatin as well as trophochromatin, as Woodruff (1921), Moore and others have suggested—seems doubtful in the light of recent investigations. Thus it has been shown by Schwartz and by Bishop that viable amiconucleate races of *Stentor* and *Oxytricha* may be derived by experimental means from normal individuals in which the idiochromatin and trophochromatin were presumably segregated in the two types of nuclei. The macronucleus of these individuals, following removal of the micronuclei, was adequate to maintain all the usual vegetative activities in the derived amiconucleate races.

In conclusion, the evidence shows that the macronucleus is the essential nuclear element in the vegetative life of ciliates. The micronucleus functions largely, if not solely, in the periodic replacement of the macronucleus and in the production of new genetic combinations, some of which undoubtedly render the species better adapted to survival. The nature of the physiological conditions which call for a renewal of the macronucleus is not clear; that such renewal meets an imperative physiological need is shown by the widespread occurrence of the phenomenon in the Euciliata.

SUMMARY

The number of micronuclei was examined in 50 trophic specimens and 50 resting cysts of each of 20 clones of *Tillina magna*, three of which were amiconucleate.

In any particular clone trophic specimens and resting cysts contained approximately equivalent mean numbers of micronuclei. In different micronucleate clones the mean number varied from 4.21 to 12.61. The mean for 1,700 specimens of 17 clones was 7.07.

The number in the individuals of any particular micronucleate clone was variable; some clones showed relatively little variation, e.g., 3 to 5 micronuclei; others, considerable variation, e.g., 2 to 11 micronuclei. The smallest number observed in any micronucleate individual was 2; the largest, 16.

All the clones produced normal resting cysts upon depletion of the food supply (*Pseudomonas fluorescens*). The cysts of different clones were equally viable and capable of excystment. Their size was unaffected by the number of micronuclei. Amicronucleate cysts showed the usual macronuclear reorganization. Hence, neither the number of micronuclei nor the absence of micronuclei affected encystment, viability and size of cysts, excystment or macronuclear reorganization.

An attempt was made to maintain each clone in pure-line culture for 60 days and thereby to examine the division rate and vitality. Four clones were refractory and encysted before 60 days expired. The remaining 16 clones, including the three amicronucleate ones, survived with undiminished vigor and were discontinued. The 13 micronucleate clones produced from 149 to 176 generations during the 60-day period; the three amicronucleate clones produced 154, 164, and 172 generations, respectively. Hence, the 16 surviving clones showed slight differences in their average daily division rates, but neither the division rate nor the vitality of these clones was correlated with variations in micronuclear number or with the absence of micronuclei. Division cysts of amicronucleate clones showed the usual macronuclear reorganization after each division of the macronucleus. The four refractory clones had high micronuclear numbers.

Since conjugation is rare and endomixis and autogamy are unknown in *Tillina*, it is probable that amicronucleate races arise at division by an unequal distribution of the daughter micronuclei.

Some of the literature on amicronucleate ciliates and on the regeneration of various types of nucleate merozoa is reviewed. The evidence shows that the macronucleus is the indispensable nuclear element in the so-called vegetative life of the organism, whereas the micronucleus during this period appears to be a relatively passive organelle. Its chief function concerns the periodic replacement of the macronucleus and the production of new hereditary combinations. Special attention is directed to the fact that inheritance in an amicronucleate race is no less precise than in a typical micronucleate race, although the division of the macronucleus is amitotic and usually reveals no suggestion of true chromosomes. It is evident that the hereditary mechanism of amicronucleate races, and perhaps of ciliates generally, differs radically from the conventional chromosomal mechanism of metazoa.

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THE EFFECT OF LOW TEMPERATURE AND OF HYPOTONICITY ON THE MORPHOLOGY OF THE CLEAVAGE FURROW IN ARBACIA EGGS¹

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When *Arbacia punctulata* eggs are exposed to low temperature during the first cleavage, a pronounced stalk develops between the daughter blastomeres. A stalk also develops at room temperature if the eggs are made to divide in hypotonic sea water or in sea water lacking calcium ion. The development of a conspicuous cleavage stalk is not a normal feature of the first cleavage in *Arbacia*, although it does occur regularly in some cells; for example, when fibroblasts divide. The object of the work reported here was to examine the conditions under which the stalk is formed in *Arbacia* and to relate these facts to current theories of the mechanism of cleavage. These particular experimental treatments were used because they were found to affect the appearance of the cleavage stalk.

METHODS

Eggs of *Arbacia punctulata* in the first cleavage served as experimental material. Ovulation was induced by the removal of the oral half of the test; eggs emerging from the genital pores were collected in a dish of sea water. The eggs were allowed to settle and the sea water was decanted after which fresh sea water was added. Two such washings were carried out to minimize contamination by coelomic fluid. Fertilization was effected by the use of diluted "dry" sperm, and the sperm were never more than one hour old.

The fertilization membranes were removed by shaking. A heavy suspension of eggs was placed in a five-inch test tube, one-half full of the suspension, and shaken rapidly thirty times. Eggs so treated cleave in time with the controls. The best time for treatment is at $2\frac{1}{2}$ minutes after fertilization, for if shaken earlier, many exovates are formed, and if shaken later, many eggs retain the fertilization membrane. The alternative method of removing the fertilization membrane by treatment with the hatching enzyme (Ishida, 1936) was not attempted.

The hyaline layer was removed in a few experiments by washing the eggs in calcium-free artificial sea water. This was accomplished by several decantations and additions of the calcium-free mixture. It was found that the hyaline layer regenerates somewhat if the eggs are returned to a solution possessing calcium ions; hence if eggs are to lack the hyaline layer, they must be allowed to cleave in the calcium-free mixture.

This study was largely accomplished by photographic means. Photomicro-

¹ This investigation was aided by a Grant-in-Aid from the Sigma Xi Alumni Research Fund.

graphs taken at intervals with Leica-lbso apparatus, were projected as negatives (1,000 \times) and measurements made with dividers.

Temperature was maintained by means of a thermostatically controlled, water jacketed, glass well, mounted on the microscope stage and connected through a centrifugal pump to a water bath. By this means temperature could be maintained within $\pm 0.2^\circ$ C. at or about 20° and within $\pm 0.4^\circ$ C. at or about 10° C.

Artificial sea water lacking calcium ions was compounded according to the method of Shapiro (1941). This solution has an osmotic pressure and pH closely similar to that of normal sea water.

The hypotonic solutions were prepared either by the dilution of normal sea water or of the calcium-free mixture.

A few observations are presented on polyspermic eggs cleaving to three or to four cells in one division. Polyspermic development was induced by the method of Smith and Clowes (1924) which involves fertilization in pH 7.2 sea water and the return of the eggs to the normal pH of 8.4 within two or three minutes.

RESULTS

Morphology of the cleavage furrow

The shape of the deepening furrow is markedly different under different conditions; it is influenced by temperature, concentration of calcium ion, tonicity and by presence or absence of the fertilization membrane.

Temperature

At temperatures between 20° C. and 30° C. there is normally no stalk in cleaving eggs whose fertilization membrane has been removed (free cleavage). The furrow is peaked at the apex (Figs. 1 and 2). At low temperatures, 6° to 12° C., a real stalk is formed during the latter part of the furrowing. This occurs whether the egg is enclosed in the fertilization membrane or not. At these low temperatures eggs undergoing membrane-free cleavage, come to resemble a dumb-bell with a handle (Figs. 3 and 4).

Calcium ion or urea

Chambers (1938) described the short cleavage stalk which develops when *Arbacia punctulata* eggs divide in calcium-free solutions at room temperature. He used isotonic mixtures of sodium chloride and potassium chloride. In the present study also a short stalk occurred when the eggs were exposed to calcium-free sea water. Similarly a short stalk was figured by Moore (1930a and b) and by Motomura (1934), after treatment with urea solutions.

Fertilization membrane

It is a common practice to remove the fertilization membrane either by dissolving it in urea solutions or by shaking an egg suspension rather violently. These techniques allow the mitotic axis to become much longer and the furrowing is thus more readily followed. If the eggs are confined in the fertilization membrane at 10° C., the blastomeres tend to stay apart and the walls of the furrow are almost vertical (Figs. 11 and 12). At the end of the cleavage a stalk connects the two blastomeres. If this same experiment is varied so that the eggs divide within their

fertilization membranes at 10° C. and in calcium-free sea water, a cleavage stalk likewise develops. In this case, however, the stalk moves eccentrically until it is close to the fertilization membrane (Figs. 30 through 34). The difference is presumably due to the fact that the hyaline layer is dissolved in solutions lacking calcium ion and when the hyaline layer is missing the egg is able to slide around inside the fertilization membrane.

Membrane-free cleavage in polyspermic eggs

Polyspermic eggs may undergo free cleavage to form four cells in the first division. In such cases they frequently divide so that a symmetrical figure is seen from above. In this circumstance the four blastomeres each rest upon the bottom of the glass container (Figs. 15, 16, 17, and 18). Frequently one blastomere rests upon the other three at the end of the cleavage (Fig. 21). The former, more symmetrical type of cleavage is more readily followed. When such an egg begins to cleave it first flattens like a biscuit; at this stage it resembles a balloon around which two rubber bands have been placed at right angles. Such a balloon is flattened on the two surfaces where the rubber bands cross. Perhaps the egg, like the balloon, is subject to greater elastic tension in the region where the incipient furrows cross, and therefore flattens on these surfaces.

As seen from above, the egg periphery is roughly square, with corners rounded (Fig. 15); the wide furrows (at 10° C.) gradually sink towards the center with the apices of the furrows approaching one another. The upper and lower surfaces meanwhile remain relatively flat although the two flat surfaces slowly come together. The whole figure at the stage illustrated in Figure 16 resembles a balloon stretched closely over four tennis balls with two rubber bands placed at right angles. Finally a definitive stalk is formed (Fig. 18).

When the furrows first appear, the egg is to be considered as having two equatorial furrows; that is, two constricting rings (Fig. 29a), which cross each other. The quasi-independence of the furrows is demonstrated by some eggs which cleave in a similar way but *in which the furrows incise at different rates* (Figs. 19 and 20). In Figure 29b the furrow separating *ab* from *cd* is well in advance of the furrow separating *ad* from *bc*. This type of cleavage leads to a figure like Figure 20.

It appears that this curious type of cleavage is brought about by the development of two new ring-like tensions which develop around the necks of the individual blastomeres after the deeper furrow is well established. As a result of the deep primary furrow, four new isthmuses are established about the necks of the four incipient blastomeres (*cf.*, Fig. 29b). Perhaps the most significant feature of

PLATE I.

- FIGURES 1 AND 2. Egg cleaving at 20° C. in sea water, fertilization membrane removed.
FIGURES 3 AND 4. Egg cleaving at 10° C. in sea water, fertilization membrane removed.
FIGURES 5 AND 6. Egg cleaving at 20° C. in 65 per cent sea water, fertilization membrane removed.
FIGURE 7. Egg cleaving within the fertilization membrane at 20° C. in sea water.
FIGURE 8. Late cleavage at 20° C. in 65 per cent sea water, fertilization membrane removed.
FIGURES 9 AND 10. Polyspermic egg fertilized in sea water at pH 7.2, transferred to normal sea water at room temperature until cleavage began. Cleaving at 10° C. in sea water.
FIGURES 11 AND 12. Eggs cleaving within fertilization membrane at 10° C.

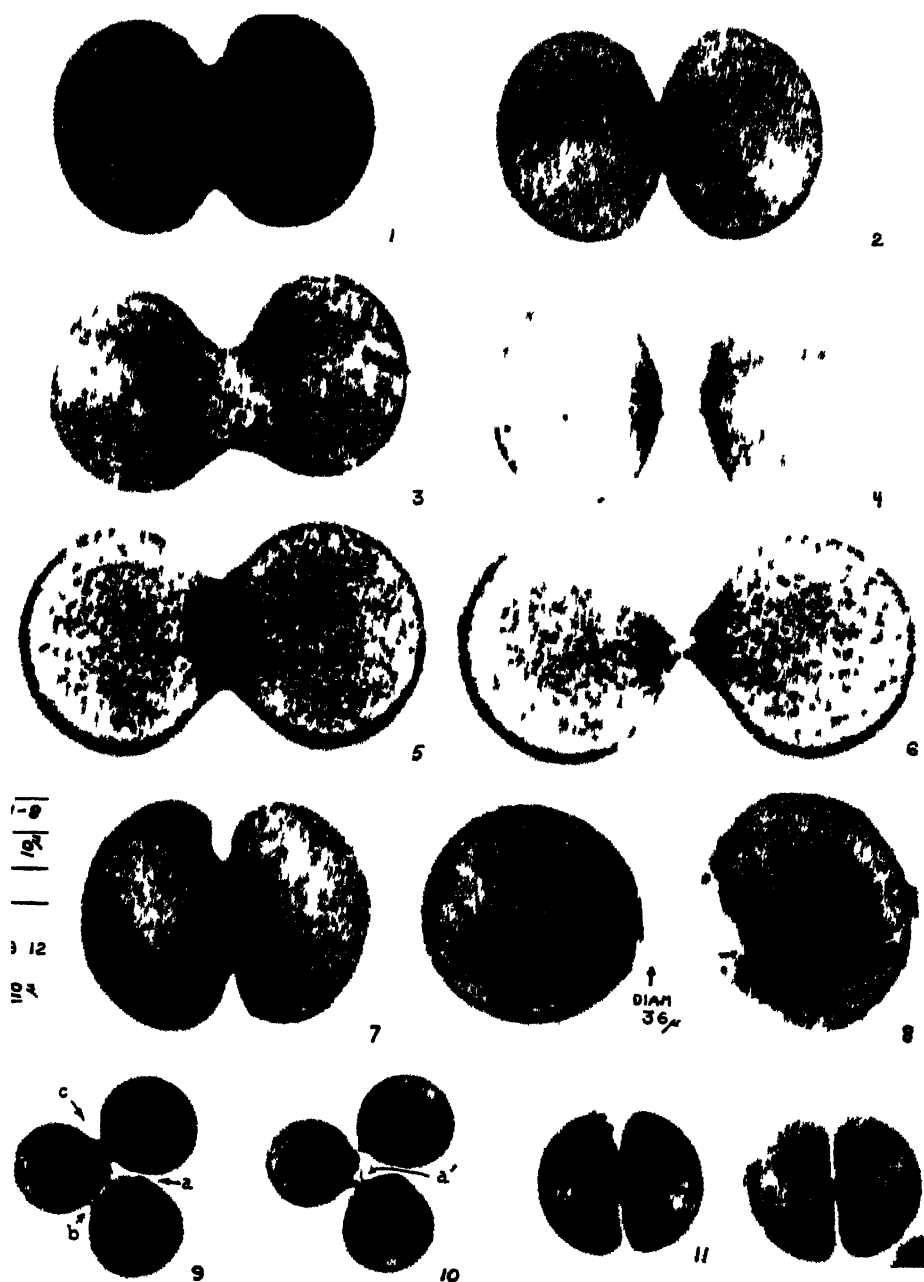


PLATE I

this type of cleavage is the bridge-like stalk which results (Figs. 23 and 24). In these latter figures note that one circumferential furrow deepened symmetrically and more rapidly than the other. The furrow which started later is very asymmetrical, being much deeper on one side (*cf.*, at the arrow) than the other. Eggs cleaving to three cells show a similar behavior (Fig. 22) and when cleavage is com-

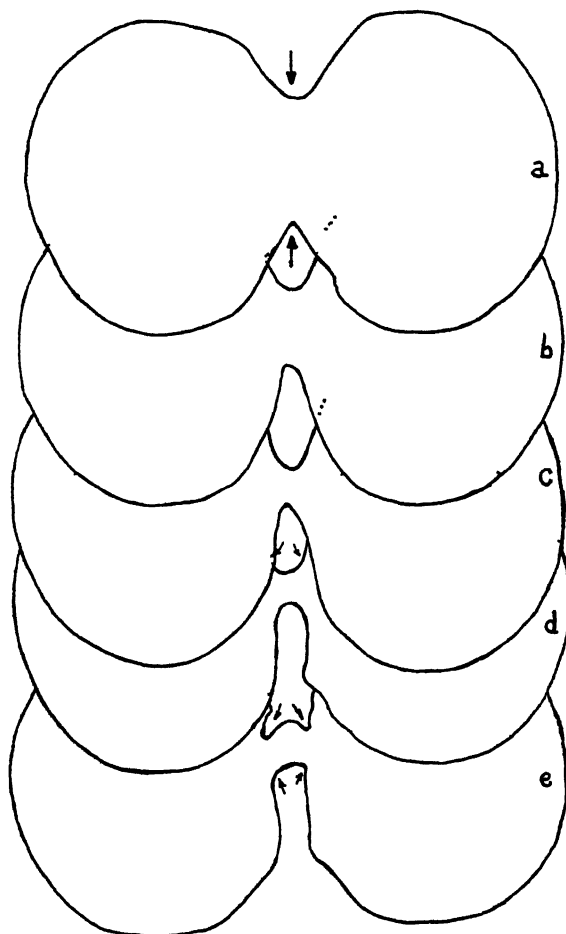


FIGURE 13. Series showing late cleavage and development of the cleavage stalk at 10° C. in sea water.

plete they may have a Y-shaped stalk, or if one furrow deepens more rapidly than the others, two stalks may connect to one blastomere (Figs. 9 and 10).

The speed of furrowing in polyspermic eggs cleaving to four cells may be as rapid as when two cells are being formed, yet it should be remembered that the amount of new surface formed is much greater when a sphere divides into four equal smaller spheres. The surface of a sphere divided into two spheres increases 26 per cent,

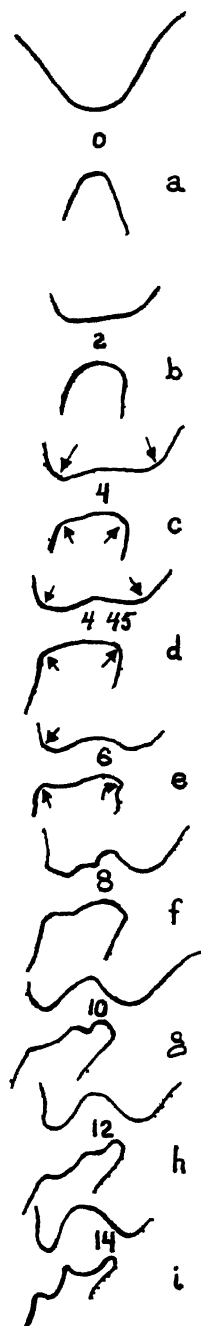


FIGURE 14. Series showing late cleavage and continued activity of cleavage stalk, during fourteen minutes in calcium-free sea water at 11° C.

if divided to four spheres the surface increases 58 per cent. A polyspermic egg cleaving to four cells forms about 26 per cent more surface than the normal first cleavage but it may do so in the same amount of time.

Membrane-free cleavage in hypotonic sea water and in hypotonic calcium-free sea water

Dilution of the sea water causes a swelling of the egg; it also causes an unusually wide furrow to develop during the cleavage and leads to the formation of a stalk at the end (Figs. 5, 6, and 8). This effect occurs at room temperature (20° C.). The stalk may become very long if the sea water has been diluted sufficiently. In mixtures of 65 parts sea water and 35 parts distilled water, for example, the stalk may finally be 30 micra long. This effect occurs either in the presence or absence of calcium ion. The stalk region is certainly a relatively rigid gel, for it has sufficient rigidity to push the daughter blastomeres far apart. Figure 8 and Figures 40 through 42 show the process of elongation in these extreme cases. Enlarged photographs of the stalk at these stages are shown in Figures 43, 44, and 45 with dimensions noted. In Figure 43 the stalk is only 4.4 micra in diameter at one point. In Figure 44 its minimum width is about 2 micra and it is over 22 micra long. In Figure 45 the constriction is completed. The stalk is still 5 micra wide at some points, but it is less than 3 micra in diameter for a third of its length. Chambers (*ibid.*) relates that a spherical oil drop lying within the egg in the furrow region is not deformed until the "external surface of the advancing furrow is 4 to 5 μ from the surface of the oil." If the egg pictured in Figure 43 has a cortex comparable in thickness, then the stalk must certainly be all gel by the time its diameter is reduced to 7 or 8 μ . One blastomere sometimes ruptures when eggs cleave in 65 per cent sea water. No endoplasm escapes if the stalk has closed. One such closed stalk is shown in Figure 28; it is 5 micra in diameter. The conclusion that the stalk is all gel (and yet it continues to constrict) is a most important conclusion for it strongly supports the contraction theory of cleavage of W. H. Lewis. Close inspection at high magnification fails to show any movement of granules located in the stalk. The active constriction of a 5 μ stalk is recorded in Figures 26 and 27.

PLATE II

FIGURES 15, 16, 17, AND 18. Cleavage of a dispermic egg, cleaving in calcium-free sea water at 10° C. Egg fertilized in pH 7.2 sea water, transferred to sea water at room temperature until beginning of cleavage. Time after fertilization: Figure 15, 72 min.; Figure 16, 74 min.; Figure 17, 88 min.; Figure 18, 190 min.

FIGURES 19 AND 20. Egg showing dispermic cleavage. Treatment as in Figures 15-18. Time after fertilization: Figure 19, 86 min.; Figure 20, 88 min.

FIGURE 21. Dispermic egg. Treatment as in Figures 15-18. One blastomere out of the horizontal plane.

FIGURE 22. Diagram illustrating two types of cleavage to three cells.

FIGURES 23 AND 24. Egg in 70 per cent sea water at 25° C., after accidental polyspermy. Time after fertilization: Figure 23, 50 min.; Figure 24, 52 min.

FIGURE 25. Dispermic egg cleaving in sea water at 11° C., following fertilization in pH 7.2 sea water.

FIGURES 26 AND 27. Late cleavage of egg in 65 per cent sea water. Room temperature. Time after fertilization: Figure 26, 83 min.; Figure 27, 85 min.

FIGURE 28. Closed stalk following rupture of one blastomere; 65 per cent calcium-free sea water.

FIGURE 29. See text.

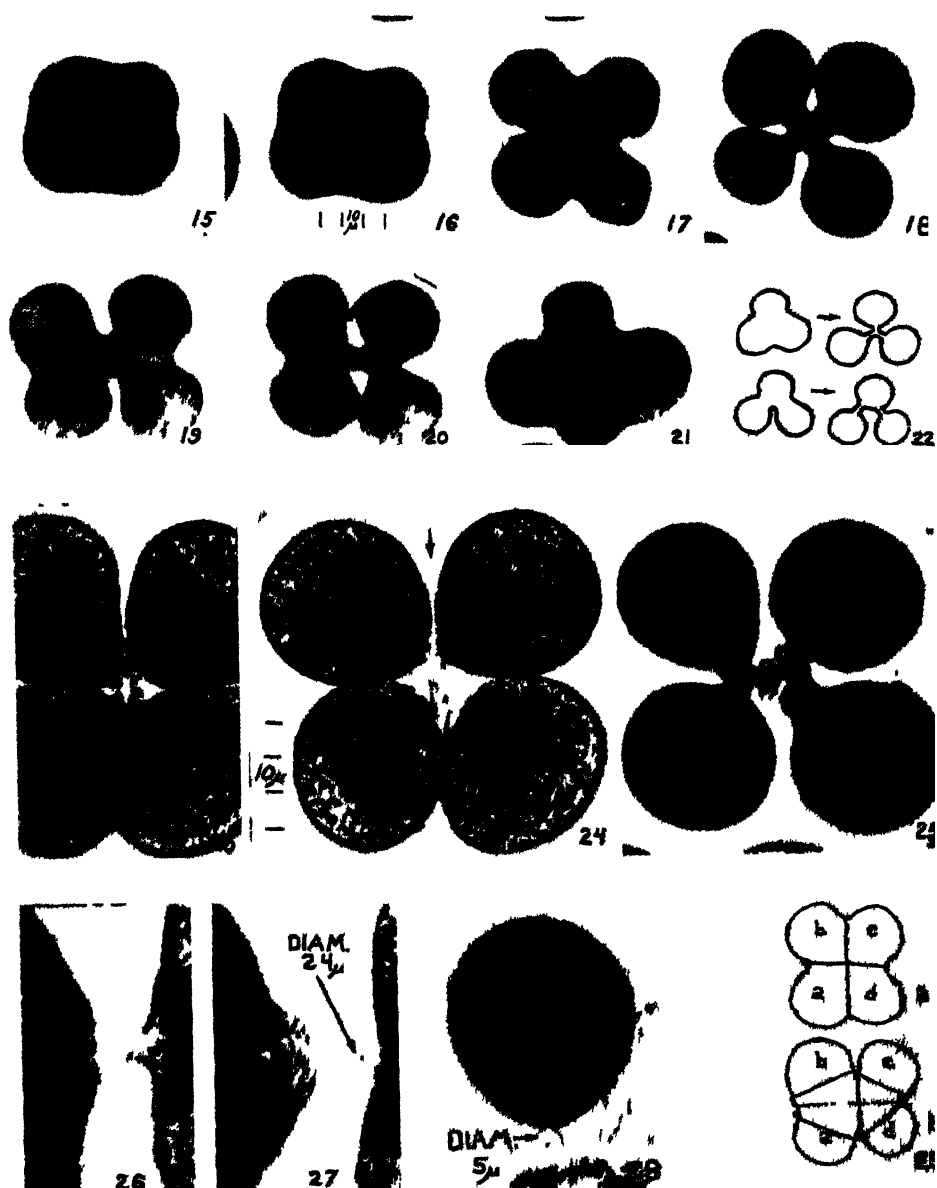


PLATE I

The stalk

The mitotic axis (greatest length) of eggs undergoing free cleavage becomes progressively longer at 10° than at 20° C. (compare Figs. 1 and 2 with 3 and 4); moreover the early furrow at 10° C. is much more blunt in contour than is the furrow of eggs at higher temperatures. A study of the final phase of cleavage under high power (Fig. 13) shows how the wide furrow is transformed into a stalk.

In Figure 13*a* the deepening furrow is still blunt with a diameter of about 14 micra. In Figure 13*b*, however, the stalk is beginning to square off. The arrows (Figs. 13*d* and *e*) indicate the region where the constriction is most active. The details are similar and are very clear in eggs cleaving in calcium-free sea water at 10° C. The series of diagrams shown in Figure 14, *a* to *i*, again show that the broad furrow first deepens until the diameter of the waist is about 7 or 8 micra (*a* and *b*), then the stalk is elongated by the constriction of the subequatorial cortex (*c* and *d*, see arrows); meanwhile the entire stalk is diminishing in diameter. The minimum diameter of the stalk is about 4 micra at 10° C. and in calcium-free sea water; in hypotonic solutions the diameter is often less. When the diameter of the stalk diminishes below 4 micra, it does so in local areas only (cf., Fig. 14*g*, *h*, *i*).

Amoeboid activity and cleavage activity

Many workers have noted that the polar surface of the cell bubbles actively during cytokinesis (Bowen, 1920—in *Euchistus spermatoctes*; and Lewis 1942—in tissue culture fibroblasts). This is not the case with the egg of the sea urchin during the first cleavage, instead the polar surface remains smooth and inactive. However, a variety of agents, will cause the formation of blebs in the sub-furrow region. One such agent is hypotonic calcium-free sea water. The blebs usually begin to form after the completion of the major furrowing and they give rise to sizable spherules which are cut off by a process very much like cleavage (Figs. 46*a*, *b*, *c*). The inactivity of the polar surface may indicate that the cortex there is different from the equatorial cortex in *Arbacia*.²

Eggs that have been in the hypotonic medium for some time may show a sudden rush of endoplasm from one blastomere to the other, often causing the blastomeres to become very unequal in size (Fig. 47 and Figs. 35 to 39).³ This endoplasmic flow is a very rapid one, usually lasting only two or three seconds. It is remarkable, however, that the flow is accompanied by a rapid deepening of the furrow, appearing as though a tension has been suddenly overcome, allowing the furrow to constrict much more rapidly than usual. The sub-cortical flow in such eggs may be down one side of the furrow, through the constricted stalk and up the other side of the furrow, yet the furrowing continues normally to completion (Fig. 47).

² The view that there is a special substance (a special type of plasmagel) located around the equator has been espoused by a number of workers, Marsland (1942) and Lewis (1942) among others. Beams and King (1937) are of the opinion that they have removed the "surface active material" of *Ascaris* eggs by centrifugation at 150,000 × gravity.

³ The rush of endoplasm described is in this case related to cleavage. It resembles the amoeboid activity described by Moser (1940) after urea treatment. Moser, p. 77, cites other cases from the literature.

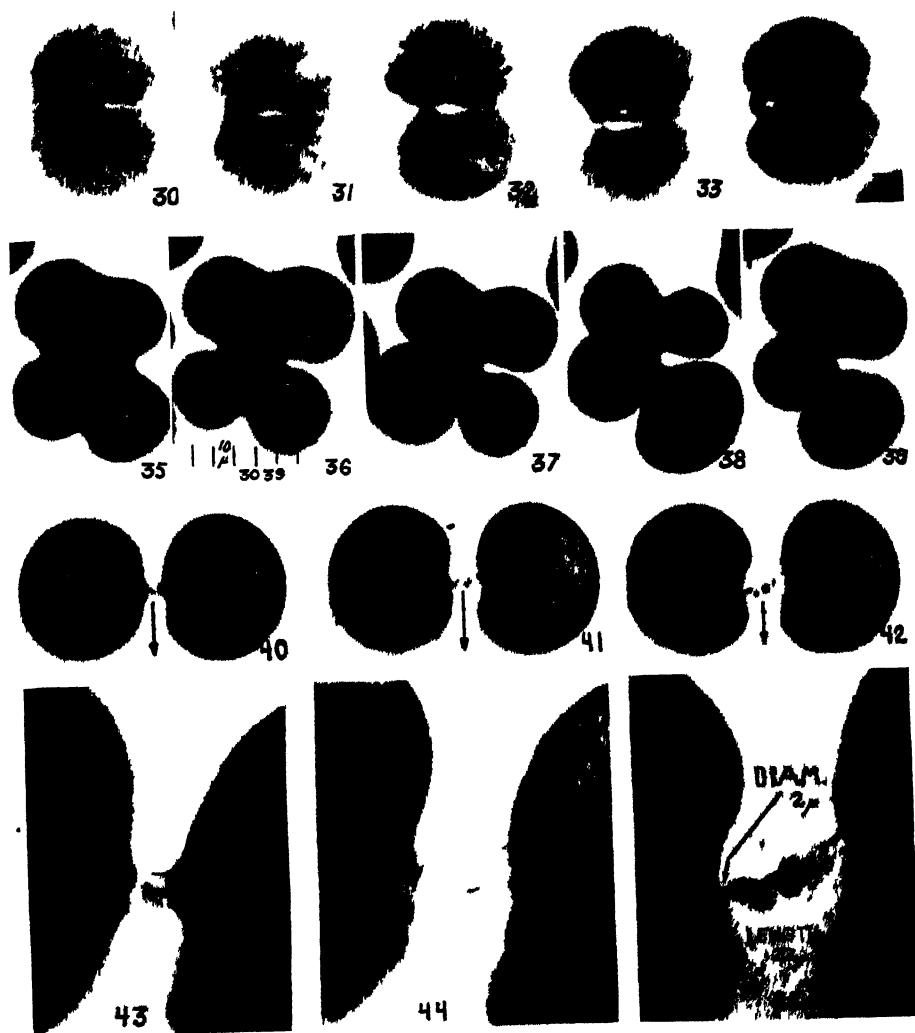


PLATE III

FIGURES 30, 31, 32, 33, AND 34. Eccentrically placed cleavage stalk Eggs in fertilization membrane at 10° C., in calcium-free sea water.

FIGURES 35, 36, 37, 38, AND 39. Volume changes of individual blastomeres Calcium-free sea water.

FIGURES 40, 41, AND 42. Elongation of the cleavage stalk; in 65 per cent sea water at room temperature. Time intervals: Figures 40-41, 1 min. and 40 sec; Figures 41-42, 1 min. and 35 sec.

FIGURES 43, 44, AND 45. Enlargements of Figures 40, 41, and 42. The edges of the nearly transparent stalk have been retouched in Figures 4, 8, 26, 27, 28, 43, 44, and 45.

DISCUSSION

The stalk

The occurrence of a stalk during the cleavage of the *Arbacia* egg is correlated with the degree of gelation of the furrow cortex. Both the observations made in this paper and those of other workers who have concerned themselves with the degree of gelation of the egg cortex confirm this. The results of several workers are summarized below:

Brown (1934): Cortical pigment granules are especially resistant to displacement by centrifugation during the division phase.

Chambers (1938): The furrow cortex resists disintegration after the two incipient blastomeres have been punctured at the poles.

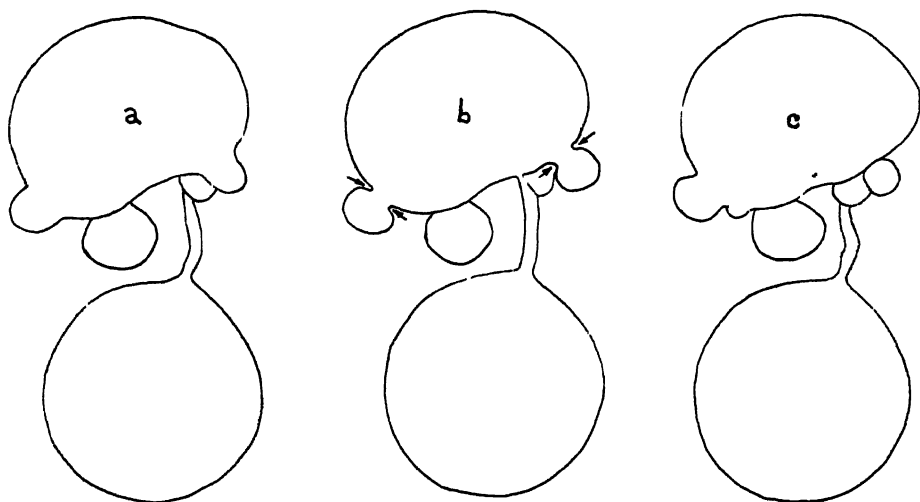


FIGURE 46

Brown and Marsland (1936): There is a quantitative decrease in the gel value of dividing eggs as the hydrostatic pressure is increased. Under high pressures the furrow regresses.

No one has yet recorded the effect of temperature, hypotonicity and lack of calcium ion upon the cortex of the dividing *Arbacia* egg, although these observations have been made upon the unfertilized egg. A brief summary of this work follows:

Costello (1938): It takes progressively longer to fragment the eggs as the temperature is lowered.

Cole (1932) and Harvey (1943): It takes longer to fragment eggs in hypotonic than in isotonic solutions.

Harvey (1945): *Arbacia* eggs break less readily in solutions possessing calcium ions than in solutions lacking calcium ions.

These treatments (low temperature, hypotonicity and calcium ion) are precisely the ones which favor the development of a cleavage stalk. It is possible that

these treatments may increase the elastic strength of the egg surface by toughening the extra cortical structures, but it is probable that they favor cortical gelation as well.

Hypotheses concerning the mechanism of cleavage; surface tension

Chambers and Kopac (1937) found that clean oil drops of the proper interfacial tension with sea water, will coalesce spontaneously with a naked egg (*Arbacia*, *Lytechinus*, and *Echinometra*). They state: "The tendency to coalescence in the furrow and polar zones of cleaving eggs (late amphiasier and later) was investigated and no difference was found." They used oils whose approximate tensions in contact with sea water were 30, 10, and 3 dynes per cm. The fact that coalescence occurs at all indicates a fluid layer around the egg periphery. Spontaneous coales-

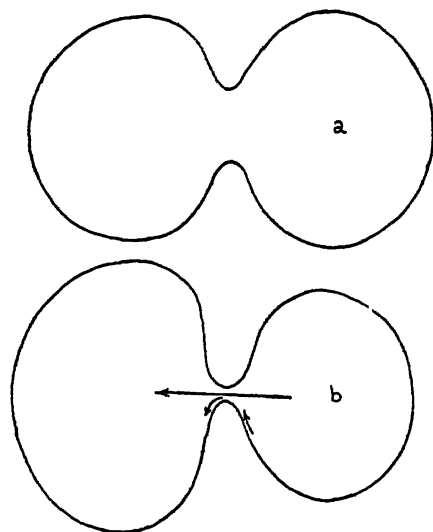


FIGURE 47

cence does not occur in *Amoeba proteus* (Kopac and Chambers, 1937), which indicates a non-fluid surface. In view of these observations any surface tension hypothesis is untenable.

Subcortical currents

Chambers (1938) has hypothesized that cleavage results from the activity of "the sub-cortical currents (which) sweep around the two asters and add gelating material to the inwardly growing cortex." In this hypothesis he combines his own observations with those of Schechtman (1937) on localized cortical growth during the cleavage of the amphibian egg. It was shown in the present paper (page 280) that normal furrowing may be associated with abnormal currents, which argues against the importance of such currents for division; moreover Lewis (1942) found no currents in the dividing fibroblast.

Astral cleavage

An astral theory of cleavage, much modified from Gray (1931), has been elaborated by Katsuma Dan (1943). He believes that the asters are composed of radiate fibers with intrinsic rigidity; he considers them to be anchored to the cortex; he believes that the rays cross at the equator; and he believes that the spindle elongates autonomously. The following quotation (Dan 1943) summarizes his theory of cytokinesis: "... it was also shown that this concept of the mechanism of cell division is adequate to explain the stretching phase of the furrow surface. That is, when two such radiate asters are pushed apart, they can in turn, push the cell membrane of the polar region somewhat as a paint brush would push some object. As they travel away, however, since they enclose the fluid endoplasm within the inter-spaces of their rays, the fluid endoplasm is carried away from the equatorial region and the cortex there is sucked in, giving rise to a furrow. The cortex is stretched as it is pulled in by the suction."

The strength of Dan's hypothesis lies in its ability to explain the differential stretching and shrinkage of the surface which he and his coworkers observed (Dan, Yanagita, and Sugiyama, 1937; Dan and Yanagita, 1938; Dan, 1943) and for which no other explanation has been forthcoming. It appears that Dan's hypothesis will explain such unusual cleavages as are pictured in Figures 9 and 10 of the present paper. It could be assumed that one element of the tripolar spindle elongated before the others causing the asters to move apart, and by the suction mechanism, causing the development of the initial furrow (Fig. 9 at *a*). On this assumption the development of the other furrows (Figs. 9*b* and *c*) begins later, presumably because the other two spindles begin their elongation later. The secondary furrow (Fig. 10 at *a'*) is presumably caused by the suction resulting from the separation of the lower two asters. Similar explanations would doubtless serve for the tetra-astral cleavages shown in Figures 23 and 24 of the present study. One can imagine also that the crossing rays from all four asters, if they became attached to the cortex, would explain the flattening of the upper and lower surfaces of the egg observed in Figure 15.

Dan's hypothesis is not in accord with the observations presented here concerning the continued elongation of the cleavage stalk in hypotonic sea water for it is impossible to see how the astral suction mechanism could explain the further constriction of a long, completely gelled stalk.

The main weakness of the astral suction hypothesis lies in its limited scope. It fails to explain undoubted cases of anastral cleavage (tissue culture, for example) frequently noted in the literature. Dan's easy conclusion that all of these anastral cases are explainable by his astral suction hypothesis ("... it is possible to imagine that in cells of the anastral type, similar gelation systems may be existing although they cannot be discerned morphologically") is unconvincing.

One of Wilson's observations is discordant with Dan's hypothesis. Wilson observed, in a form which normally has asters, that a spindle need not be present for complete cleavage to occur. He found that a cleavage furrow may cut in around the base of an isolated aster and result in a complete cleavage. Compare Wilson (1901), page 376 and Figure 11.

In one of Chamber's microdissection experiments he bisected the partially cleaved egg in a plane at 45° to the plane of the furrow (1924, Fig. 36). The cut resulted

immediately in two cells. However, the original furrow remained on each artificially produced blastomere and, on each, the furrow gradually cut through forming two small "cells" as well as two large ones. This continued cleavage seems to be quite unexplainable by Dan's hypothesis which requires crossed astral rays, an elongating spindle and a suction produced by the separation of the asters.

Cortical growth or cortical contraction?

Schechtman has proposed another theory of the mechanism of cytokinesis. He suggested (1937) that the furrow cortex grows by the "intussusception of clear cytoplasm," but simple growth of the equatorial cortex would not be expected to cut the egg in half. Other factors must account for the inwardly directed furrow and its narrowing. It seems clear that there is a stretching of the egg cortex at the time of furrowing as concluded by Dan et al. (1937, 1938), by Schechtman (1937) and by Motomura (1940), but whether the stretching is active (the result of growth) or whether it is passive and due rather to a contracting ring at the head of the furrow (Lewis, 1942), is not easy to decide. Schechtman is of the opinion that "Cleavage is initiated by a contraction of the egg cortex at the site of the future furrow." And he notes that the "Cortex becomes thicker and bulges toward the egg interior." He therefore uses both contraction and cortical growth in his complete hypothesis. The observations made in this paper on the continued constriction of small stalks after they consist entirely of gelated material are taken as strongly favoring the constricting ring theory of Lewis. For if the gelated stalk is able to contract at that late stage of cleavage, it seems reasonable to suppose that it possesses contractile power earlier. The direction of contraction is ringwise about the equator (Fig. 29a) and it is to be expected that such contraction would draw stained areas out into fine lines as Schechtman observed, if such areas are located in the furrow or subfurrow region.

It would be illuminating to know whether or not kaolin particles placed *around* the equator would be brought closer together during the furrowing but no one has made these observations.

Amoeboid activity and bleb formation

One can scarcely observe the amoeboid behavior of eggs in hypotonic media and particularly the "normal" false cleavages which occur during the amoeboid phase preceding pronuclear fusion in the nematode egg (Spek, 1918), without being convinced that a fundamental similarity exists between amoeboid motion and cleavage. Moreover the abscission of blebs is strikingly similar to cleavage.* It is suggested that any deforming force which establishes an isthmus about the cell or a part of the cell will result in the development of a contracting ring disposed around the isthmus, provided that the egg is in the cleavage phase. This view would explain why the normal egg, deformed by the elongating spindle, cleaves at the equator. It would explain why cleavage planes cut in around the base of cytasters which are unconnected to a spindle (Wilson, 1901) and it would explain why blebs formed in

*Very recently Holtfreuter (1946) has suggested "that in normal cytoplasmic division the activity of the nucleus and of the endoplasm are of a mere secondary importance." He observed that isolated, embryonic amphibian cells may develop annular constrictions which lead to the fragmentation of the cell. He considers, however, that the contraction occurs in the membrane rather than in the plasmagel layer.

the sub-furrow region may cut off from the remainder of the egg as reported above. This hypothesis also agrees with the idea that the enlarging gelated asters play a mechanical role in localizing the furrow.

SUMMARY

1. Under certain conditions the eggs of *Arbacia punctulata* develop a cleavage stalk between the first two blastomeres. No stalk forms in sea water if the temperature is in the 20° C. to 30° C. range; low temperature (10° C.) causes the development of a stalk in sea water; a short stalk develops in isotonic calcium-free sea water at 20° C.; a very long stalk develops if eggs are cleaving in hypotonic sea water (65 per cent).

2. The effect of the above treatments on the appearance of cleaving dispermic eggs is described.

3. Evidence indicates that stalks of 8 micra diameter are all gel, yet in hypotonic sea water they continue to constrict and elongate. This is good evidence that the cortical gel has inherent contractile properties.

4. It is hypothesized that any event which deforms the *Arbacia* egg (if it is in the "cleavage phase") leads in some way to an orientation of contraction around the isthmus. The deforming force may be an enlarging aster, an elongating spindle, or an endoplasmic flow.

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DEVELOPMENTAL RELATIONS BETWEEN GENITAL DUCTS AND GONADS IN *DROSOPHILA*

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While studying the reproductive system of *Drosophila simulans* gynandromorphs Dobzhansky (1931) made the following interesting observation: If female genital ducts and testes were present in the same individual and if the female ducts were attached to the testes, the latter underwent extreme degeneration. Yet, the attachment of male genital ducts to ovaries did not affect the development of these organs.

Now we know that in normal development the attachment of the ducts to the gonads takes place during the early period of pupal life. We know further that by transplantation of gonads from one individual to another it is possible to obtain attachment of the transplanted organ to the host ducts. This knowledge makes it possible to attack experimentally the question whether the degeneration of testes when attached to the female ducts, as observed in *Drosophila simulans* gynandromorphs, is a peculiarity of this special case, or whether the phenomenon is a general one and always occurs when female ducts and testes are brought into contact with each other.

The problem can be approached experimentally in two ways: the larval testes can be transplanted into female host larvae or the female genital disc from which the ducts originate can be transplanted into male larvae. By transplanting two or three testes into one host, the chance that one transplant will attach itself to the host duct is quite good. The chances for attachment of the testes to the female duct are even better when the six oviducts which arise by outgrowths from the three transplanted imaginal discs compete with the two host ducts for attachment. In the following studies both these methods were used.

EXPERIMENTAL

Transplantation of testes into female ducts

Two or three testes of mature virilis larvae were transplanted together into the abdominal cavity of female host larvae of the same age. After the hosts had emerged, the condition of the transplants and their relationship to the female genital system was studied in careful dissections. This series consisted of ten cases. The following was found. All transplants had failed to assume their characteristic spiral shape. This was to be expected, since the work of Dobzhansky (1931) and Stern (1941a and b) had shown that the testes have to be attached to the vas in order to accomplish their spiral growth. In six out of ten cases one of the transplanted testes had attached itself to one of the oviducts of the hosts. The attached testis was always greatly reduced in size and appeared degenerate. Figure 1 shows camera lucida drawings of five representative cases of this series. It will be noted that the degenerative reduction occurs only when the testis is attached to the *oviduct* of

the host (Fig. 1*A, B, D* and *E*). Testes that lie free in the body cavity (Fig. 1*A* and *B*) or testes that have been attached to the ovary (Fig. 1*C* and *B*) are unaffected. Thus the principle which produces degeneration is apparently given off only by the oviducts and depends for its action on a close cellular contact with the testes. This principle, moreover, seems unable to penetrate larger cell barriers, for testes which were connected to ovaries which in turn had their normal oviduct connection remained unaffected (Fig. 1*C*). Yet two testes which had established close

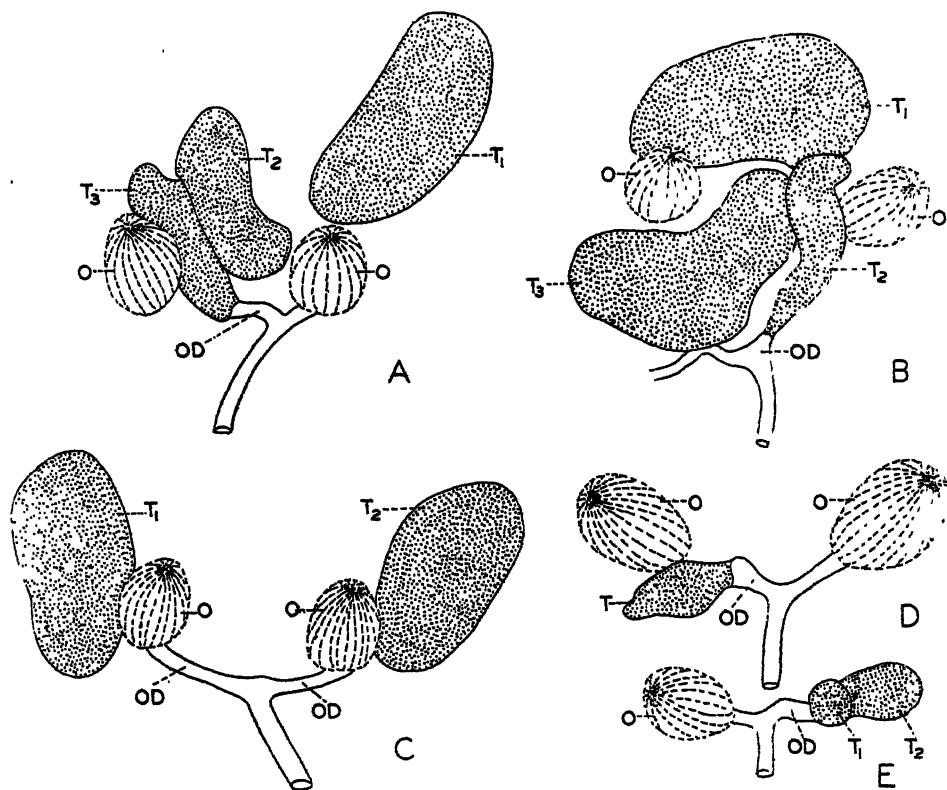


FIGURE 1. The spatial and developmental relations of transplanted testes to the reproductive system of their female hosts. *O*, ovary; *OD*, oviduct; *T*₁ to *T*₃, transplanted testes.

contact with each other had both suffered degenerative reduction, although only one of these fused organs has actually established contact with the oviduct (Fig. 1*A* and *E*).

Transplantation of female genital discs into male hosts

In a second series of experiments, two or three female genital discs from mature virilis larvae were transplanted together into the body cavity of hosts of the same age. The condition of the host testes and their relationship to the transplanted female structures was again studied by dissection. Several of the affected testes

were also sectioned and studied histologically. Thirty successful cases were available for investigation. In seven of these cases, the host testes were not connected to the transplanted ducts, although the latter had developed well and were found in the immediate neighborhood of the male gonads. The testes of these seven hosts were normal in size, shape, and histology. One testis in each additional animal was not connected with the vas of the host, nor to any of the transplanted ducts. These testes were not coiled but were otherwise normal. The other testis in two of these individuals was connected to the vas of the host and was normal, while the other testis of the third individual, although connected to the vas, was not coiled. The non-coiling of an attached testis is rare, but has been observed at times in otherwise normal animals. Whether the inability of attached testes to coil is a result of faulty connections with the vas or whether the vas in these cases has lost its growth inducing capacity is not known.

TABLE I

Transplantation of female genital ducts into male hosts

Number of discs transplanted	Testis free (round)		Testis (spiral). Normal attached		Testis attached to ♀ duct		State of degeneration of testis when attached to ♀ duct	
	One side	Both sides	One side	Both sides	One side	Both sides	One side	Other side
3			Yes		Yes		+	
3			Yes		Yes		++++	
3			Yes		Yes		++++	
3			Yes		Yes		++++	
3				Yes				
3				Yes				
3			Yes		Yes		++++	
2			Yes		Yes		++++	
2			Yes		Yes		++++	
2						Yes	+++++	+++++
2	Yes		Yes				+	
3			Yes		Yes		++	
3				Yes				
3			Yes		Yes		++++	
3			Yes		Yes		++++++	
3			Yes		Yes		++++++	
2				Yes				
2			Yes		Yes		+++	
2		Yes						
2		Yes						
2		Yes						
2				Yes				
2				Yes				
2						Yes	+++++	+++++
2			Yes		Yes		++++	
2						Yes	+++	+++++
2 h.				Yes				
2 h.			Yes		Yes		++++	
2 h.			Yes		Yes		++++	
2 hv.			Yes		Yes		++++	

h. = hydei discs into hydei hosts. hv. = hydei discs into virilis host. * = testis attached to vas, but not spiral.

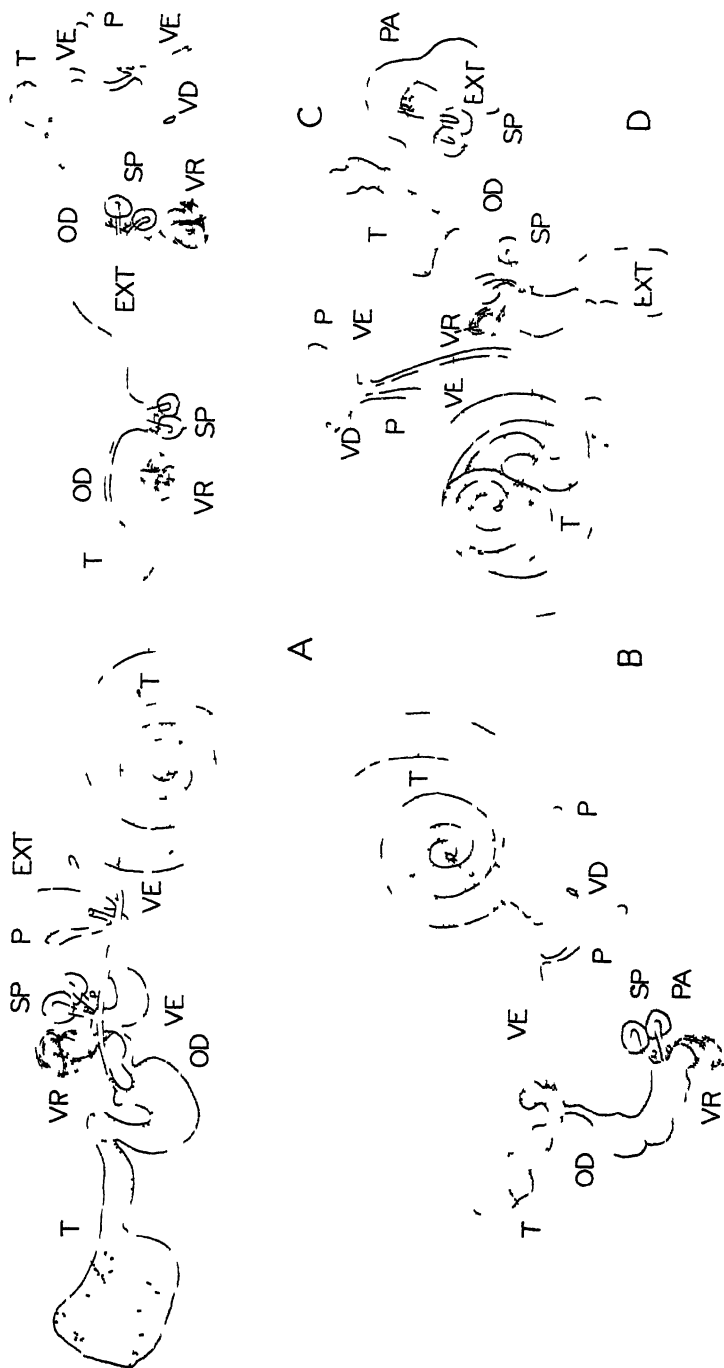


FIGURE 2 The spatial and developmental relations of transplanted female genital structures to the reproductive system of the male hosts. *EXT*, external female genitalia formed by the transplants; *OD*, oviduct; *P*, paragonum; *SP*, spermatheca; *T*, testis; *VD*, vas deferens; *VR*, vas efferens; *VE*, ventral receptacle.

In twenty individuals one or both testes were connected to the oviduct of the transplant. In some cases one testis was even found to have connection with the oviducts of two discs. All testes that were attached to oviducts showed a more or less pronounced degree of reduction and appeared degenerate. Table I summarizes the results of this experimental series. The state of degenerative reduction of the testes in this table is indicated by crosses. One cross signifies slight; six crosses, extreme size reduction. Figure 2 shows camera lucida drawings of four representative cases of this series. Figure 2*A* is a case listed in Table I having one cross. Figure 2*C* is listed by having six crosses and Figure 2*D* and *B* are listed by having five crosses each. Figure 3 illustrates by microphotography an extremely reduced testis.



FIGURE 3. *A*, normal adult testes. *B*, (arrow) testis of the same individual degenerated under the influence of a transplanted and attached oviduct.

It will be noted from Table I that the reduction of the testes is in most cases very pronounced. Only six of the testes attached to oviducts were reduced to state "3" or less, while sixteen testes were degenerated to state "4" or more.

This experimental series thus confirms strikingly the original observation that testes attached to female oviducts suffer degenerative changes and that it is the oviduct that elicits the principle causing degeneration. The observation from the previous experiments that the presence of oviducts *per se* has no effect on testis development is also confirmed, for testes in the presence of as many as three pairs of oviducts in the immediate organic environment remained normal if they were not attached to the oviduct. In comparing the attached testes of the two series with each other, a difference in their general shape was noted. While the transplanted

testes in the first experimental group were small, roundish bodies, the testes in the second experimental group were in most cases thin and elongated in shape (compare Fig. 1 with Fig. 2). Now it was found in the second group that in all cases when the attached testis was thin and elongated it was attached not only to the transplanted oviduct but also to the vas efferens of its host (Fig. 2). In those cases, however, where the testis attached only to the transplanted oviduct, had not established connection with the vas, it was roundish. This situation is well illustrated in Figure 2C. The left testis in this case, a small roundish degenerated organ, is attached only to the oviduct while the right testis, which is attached to the vas efferens of the host and to one transplanted oviduct, has become an irregularly elongated structure. The elongated shape of such a degenerate testis is thus due to the stimulating influence of the vas on the growth of the testis, which in normal development leads to the coiling of this organ, while the observed degeneration is caused by the influence of the oviduct.



FIGURE 4. Sections through two extremely reduced testes. *d*, degenerating cells.

Not only virilis but also hydei oviducts cause degeneration of hydei testes attached to them. This was shown by three cases in which larval hydei female discs were transplanted into hydei male larvae (see Table I).

The factor in the oviduct causing degeneration of the testis by contact is not species specific, for hydei oviducts will cause virilis testes to degenerate (see Table I).

Sections of reduced testes were made and their histology studied. It was found that, depending upon the degree of reduction of size, the testes contained various amounts of spermatogonia and spermatocytes in all stages of degeneration. The remnants of disintegrated cells in the form of granular picnotic masses together with quite normal appearing cells were observed. Figure 4 shows the condition found in an extremely reduced testis.

CONCLUSION AND SUMMARY

By transplanting female genital discs into male hosts, attachment to the host testes of oviducts developed from transplanted genital discs is obtained. In these cases the attached testes suffer extensive degeneration. Only cellular contact of

the oviducts to the testes brings about this phenomenon. Unattached female ducts do not affect the development of the testis. The principle causing degeneration is not species specific. The findings indicate that the phenomenon encountered is no unique instance, but representative when oviduct and testis establish cellular contact during pupal development.

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A HISTOLOGICAL STUDY OF SYNDISYRINX FRANCISCANUS,
GEN. ET SP. NOV., AN ENDOPARASITIC RHABDOCOEI
OF THE SEA URCHIN, STRONGYLOCENTROTUS
FRANCISCANUS¹

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INTRODUCTION

Up to the present time eight genera of worms endoparasitic in echinoderms and sipunculids have been described that belong to the rhabdocoel family Umagillidae Wahl, 1910b. Schneider described the first species, *Anoplodium parasita*, in 1858. Since then six questionable and three valid species of this genus have been reported from widely separated localities as parasites of holothurians. Their distribution extends from the Mediterranean, Ionian, and North Seas to Japan and the Philippines (Bock, 1926). *Syndesmis echinorum* Francois, 1886, the only species of the genus, is found in echinoids. It has been collected in the Mediterranean (Russo, 1895), Norway (Westblad, 1926), and the English Channel (Braun, 1889). Three species of the genus *Collastoma* are found in sipunculids at Roscoff (Dörler, 1900), the Gulf of Kola (Beklemishev, 1916), and the Bay of Naples (Wahl, 1910a). The genus *Desmote* is represented by one species, *D. vorax*, discovered in a crinoid collected in the Gulf of Kola (Beklemishev, 1916). A single species parasitic in holothurians has been described in each of four genera, i.e., a Japanese form, *Xenometra arborea* Ozaki, 1932, and three reported from the coast of Norway, *Wahlia macrostylifera* Westblad, 1930, *Anoplodicra voluta* Westblad, 1930, and type genus *Umagilla forskalensis* Wahl, 1909.

The only reference to a member of the Umagillidae from the Western Hemisphere was made by Powers in 1936. He reported the presence of a Syndesmis-like worm in the coelomic cavity of the echinoid, *Centrechinus antillarum*, at Tortugas. A complete description was not given; however, as compared with *Syndesmis*, noticeable differences were observed in details of the copulatory apparatus and the arrangement of the shell glands. While the endoparasitic rhabdocoel of *Strongylocentrotus franciscanus*, the large common sea urchin of the California coast, is well known to some investigators who have worked at Pacific Grove, a description of this worm has not been recorded in the literature prior to the present account.

¹ This work was done at the Wilson Zoological Laboratory of the University of North Carolina in partial fulfillment of the requirements for the degree of Master of Arts. The author is indebted to Professor D. P. Costello for suggesting the problem, for the slide preparations upon which this study was essentially based, and for the invaluable suggestions and criticisms rendered during the preparation of this paper. The author wishes to acknowledge his appreciation to Dr. L. H. Hyman for many valuable recommendations and for permission to introduce her revised and hitherto unpublished terminology relating to this group. To Miss Catherine Henley the author expresses his gratitude for the translation of a number of the references cited herein.

² Now in the School of Biological Sciences of Stanford University.

Systematic position
Order Rhabdocoela
Suborder Lecithophora
Section Dalyellioida

Family Umagillidae Wahl, 1910
Subfamily Umagillinae Wahl, 1910

Genus *Syndisyrinx*, gen. nov.
(Genotype *Syndisyrinx franciscanus*, sp. nov.)

Holotype. A whole mount in the United States National Museum, Washington D. C.

Repositories of type material. In each of the following repositories a whole mount, a transversely sectioned, and a sagittally sectioned preparation selected from the type material have been deposited: U. S. National Museum, Washington, D. C.; American Museum of Natural History, New York City; British Museum, London; California Academy of Science, San Francisco; Wilson Zoological Laboratory of the University of North Carolina; and Museum of Natural History, Stanford University. Additional preserved material may be obtained from the author or from any of these institutions.

Type locality. Mussel Point, Monterey Peninsula, California, Lat. 36°, 37' 20" N., Long. 121°, 54', 15" W.

Collectors. D. P. Costello, 1937 and H. E. Lehman, 1945.

Distinguishing characteristics. Umagillinae with a single intestine, paired and branched ovaries, cuticular penis, and a bursa seminalis connected by cuticular ducts to the seminal receptacle and bursal canal.

MATERIALS AND METHODS

Fifty-four rhabdocoel parasites were obtained from two specimens of the sea urchin *Strongylocentrotus franciscanus* (A. Agassiz) by Dr. D. P. Costello in August 1937 at Pacific Grove, California. These specimens were fixed in Heath's, Boveri's, Lillie's and Worcester's solutions. Five of the individuals were sectioned serially at 10 μ and stained with Heidenhain's iron hematoxylin and orange G. One of these preparations was exceptionally fine and the majority of the accompanying figures were made from it. Unfortunately this preparation, which the author intended to designate as the holotype, was lost when a microscope was stolen. This material, including the slide preparations, was turned over to me by Dr. Costello. The morphological study was based on this material.

In the summer of 1945 during June, July, and August, the author collected several hundred additional specimens from the same locality. Over sixty urchins were examined and all were found to be infested; frequently three dozen or more parasites were obtained from the intestine of a single host. These worms were fixed in Heath's and Beauchamp's solutions. Seventy were sectioned serially at 10 μ and stained with Mayer's acid hemalum and triosin. Thirty whole mounts stained with paracarmine were also made. The type material was selected from these prep-

³ Classification according to Bresslau (1933), with the exception of "Family Anoplodiidae Graff, 1913," which has been rejected in favor of "Family Umagillidae Wahl, 1910b," inasmuch as no reason is given by Graff for discarding the older name or for selecting *Anoplodium* as type genus. The subfamily Umagillinae has been retained as designated by Wahl, 1910b

parations. At this time another parasite of *Str. franciscanus* was discovered which differed from *Syndisyrinx* in shape, manner of locomotion, and color. A description of this worm is being prepared and preliminary examination of sectioned material indicates a close relationship to *Syndesmis echinorum*. Upon the suggestion of Prof. A. R. Moore, who had occasionally observed parasitic worms in *Str. purpuratus* (Stimpson), forty-seven of these urchins were examined. In twenty-nine of them, worms that are very similar to, and may be identical with *Syndisyrinx franciscanus* were present in small numbers.

GENERAL MORPHOLOGY

The living animals are bright red with a dark brown or yellow median longitudinal line which marks the extent of the intestine. The worms are flattened dorsoventrally and have a leaf-like appearance, being rounded at the anterior end and slightly pointed posterad. Individuals vary in size from 2 to 3 mm. long and 1.6 to 2.5 mm. wide. The body is thickest at approximately one-fourth of the distance from the anterior end and at this level measures about 0.5 mm. in the dorsoventral axis. Laterally and posteriorly the thickness of the body diminishes gradually to about 0.2 mm. at the periphery. A ciliated epithelium covers the entire surface; rhabdites and cuticle are lacking.

The mouth is situated on the ventral surface about one-fourth of the distance from the anterior end and a common genital pore opens ventrally at the posterior extremity of the body. The musculature and parenchyma are typical of other Umagillidae. No excretory system was observed. The strongly muscular pharynx is typically doliiform and possesses pharyngeal glands; it communicates by a short oesophagus with the gut. The intestine, possessing a number of small lateral diverticula, extends posterad under the dorsal epidermis along the mid-line and terminates one-quarter of the distance from the posterior end of the body. The gut contains no permanent lumen and food masses lie in temporary cavities surrounded by large digestive cells. The brain, composed of two cerebral ganglia connected by a wide commissure, lies anterior to the pharynx and gives off paired anterior, lateral, and posterior nerves.

Lobed testes lie lateral to the mid-line in the anterior half of the body. Accessory glands empty into the sperm duct that arises from each testis and passes anterad. These paired tubes unite mesially and enter a small spermiducal vesicle that is continued posterad as a muscular common sperm duct which lies dorsal to the uterus along the mid-line. This tube terminates in an elongated cuticular stylet, the penis, which is enlarged and funnel-like at the base. The penis stylet enclosed in the male antrum extends through the posterior third of the body to the common genital antrum and over most of its length does not exceed 3μ in diameter.

Paired vitellaria are found immediately posterior to the testes; they are greatly ramified and fill most of the ventrolateral spaces in the middle third of the body. Posterior to the vitellaria a pair of ovaries is located, one on each side of the mid-line. Laterally each branches into five or more finger-like lobes. Three or four collecting ducts from the vitellaria empty with the ovaries and seminal receptacle into the anterior end of the ovovitelline duct. The seminal receptacle is oval and filled with sperm. Located posterodorsad to this organ is a vesicular, sperm-filled bursa seminalis connected to the seminal receptacle by a fine cuticular insemi-

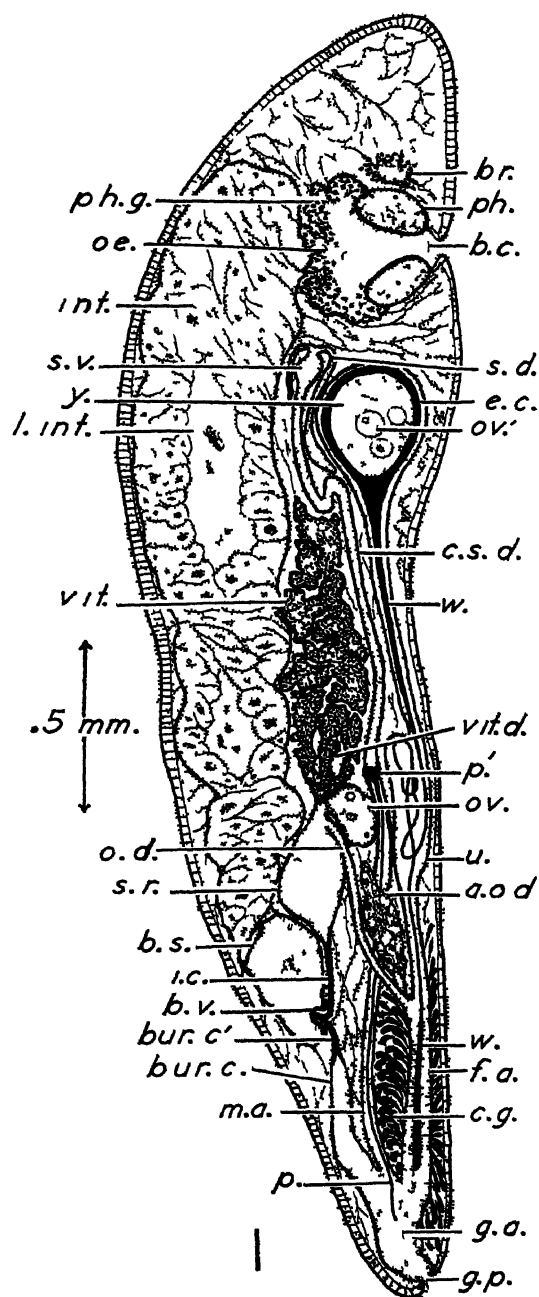


FIGURE 1 Semidiagrammatic median sagittal section

nation canal. Arising in close association with this tubule is a similar duct, the cuticular proximal part of the bursal canal that passes posterad from the bursa seminalis approximately 60μ before widening into the posterior muscular portion of the bursal canal (vagina). A cuticular sheath surrounds the openings of these two ducts into the bursa seminalis. The composite structure, consisting of this sheath and the canals passing through it, makes up the bursal valve.

An ovovitelline duct, into which accessory glands empty, arises ventrally at the anterior end of the seminal receptacle. It passes posterad and unites with the female antrum. The uterus, lying close to the ventral epidermis, extends anteriorly from the female antrum almost to the pharynx. At the anterior end of the uterus an egg capsule containing from one to five ova and numerous yolk cells is generally found. The capsule is continued posterad as a long coiled whip similar to those found in related forms. Most of the ventrolateral spaces of the posterior third of the body are filled by cement glands; they communicate by many small ducts with the female antrum. The common genital antrum is an elongated cavity at the posterior end of the body into which the female antrum enters ventrally, the male antrum and penis open mesially and the bursal canal is given off dorsally. At its posterior end is the common genital pore which opens ventrally to the exterior.

HISTOLOGICAL STRUCTURE

Epidermis

A ciliated epithelium covers both dorsal and ventral surfaces of the body. No pigment or special gland cells were observed in this layer and a cuticle and rhabdites are lacking. The cytoplasm of the cells in the epidermal layer is granular and cell boundaries, though faintly stained, are distinct. The cells covering the dorsal surface are cuboidal and measure 10μ from basement membrane to external surface. The cytoplasm of these cells stains moderately with hematoxylin. On the ventral surface the cells are flattened and are about 7μ thick and from 12 to 35μ wide; they have little affinity for hematoxylin. Cilia of the ventral epidermis are about 6.5μ long and are almost twice the length of those found on the dorsal surface. Cells possessing the staining properties and short cilia characteristic of the dorsal layer extend for a short distance ventrally around the lateral edges. A zone 4 to 6 cells wide of intermediate nature accomplishes the transition between typical dorsal and ventral epithelium.

Musculature and parenchyma

The arrangement of the musculature is essentially the same as that described for other Umagillidae. Under the basement membrane of the surface epithelium is

Abbreviations for Figures 1 and 2.

a.o.d.—accessory glands of ovovitelline duct, a.s.d.—accessory glands of sperm duct, br.—brain, b.c.—buccal cavity, bur. c.—bursal canal, bur. c'.—cuticular end of bursal canal, b.s.—bursa seminalis, b.v.—bursal valve, c.g.—cement glands, c.s.d.—common sperm duct, e.c.—egg capsule, f.a.—female antrum, g.a.—common genital antrum, g.p.—genital pore, int.—intestine, i.c.—insemination canal, l. int.—lumen of intestine, m.a.—male antrum, oe.—oesophagus, ov.—ovary, ov'.—ovum, o.d.—ovovitelline duct, p.—penis, p'.—base of penis, ph.—pharynx, ph. g.—pharyngeal glands, s.d.—sperm duct, s.r.—seminal receptacle, s.v.—spermiducal vesicle, te.—testis, u.—uterus, vit.—vitellaria, vit. d.—vitelline ducts, w.—whip of egg capsule, y.—yolk cells.

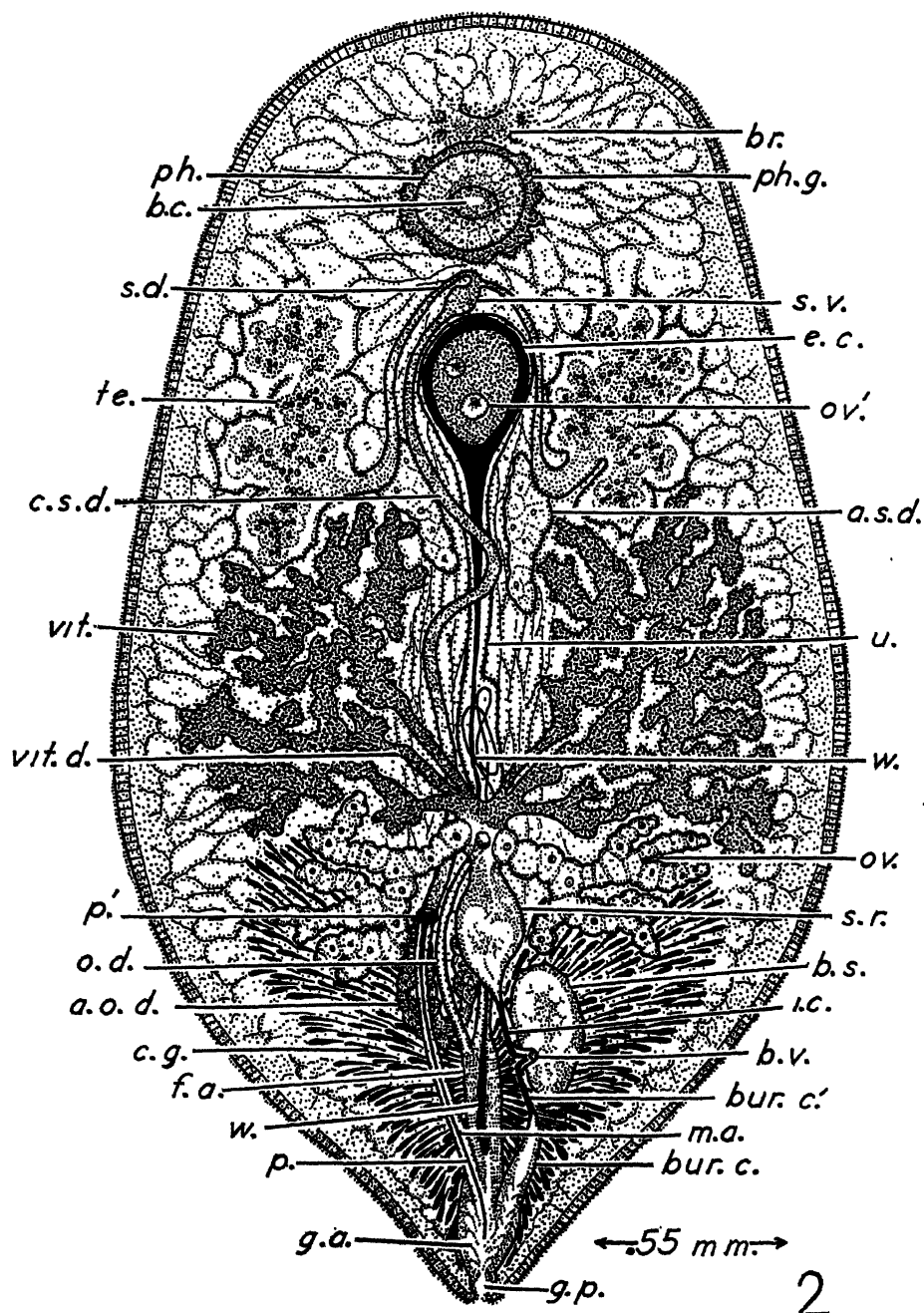


FIGURE 2. Semidiagrammatic median frontal section, intestine omitted.

found a thin layer of subepidermal muscles (Figs. 3-5, 7, 8). The superficial muscles are circular; these overlie a longitudinal sheet, and interposed at intervals between these layers are well-developed oblique fibers. In addition to these, bundles of fibers attached to the internal organs or the basement membrane of the epidermis pass dorsoventrally through the parenchyma (Figs. 1-3). The special muscles of the reproductive and digestive systems will be described in connection with the organs with which they are associated.

A parenchyma, composed of large, irregularly shaped cells with coarsely granular or vacuolated cytoplasm, fills most of the spaces between the internal organs and epidermis. A histologically distinct parenchymatous mass of cells enclosed in a fibrous capsule extends posterad along the mid-ventral line from the posterior level of the pharynx to the region in which the female antrum enters the common genital antrum. The flattened, nonvacuolated cells of this tissue possess finely granular cytoplasm and are arranged in concentric layers around the reproductive ducts, most of which pass through the mid-ventral parenchyma (Figs. 3-5, 7). Nowhere within the parenchyma were flame cells or collecting ducts of an excretory system observed.

Nervous system

The brain is similar in all respects to those described in other members of the family. It is located just anterior to the pharynx and consists of two ganglia connected by a wide commissure. Around the central fibrous mass of the brain are numerous ganglionic cells that stain quite evenly with hematoxylin. Poorly developed anterior, lateral and posterior pairs of nerves leave the brain and can be traced for short distances into the parenchyma. No theca separates the brain or nerves from the parenchyma and no special sensory organs were found.

Digestive system

The mouth lies on the ventral surface about one-fourth of the distance from the anterior end of the body. It opens into a very small buccal cavity lined by flattened ciliated cells that are continuous externally with the ventral epithelium (Figs. 1, 8). A sphincter underlying the epithelium regulates the size of the oral opening. Lying immediately dorsal to the mouth and opening into the buccal cavity is the doliiform pharynx which has the appearance of a dorsally compressed sphere. Its dorso-ventral axis is about 0.1 mm. long and its greatest diameter is about 0.17 mm. Passing dorsoventrally through the pharynx is a funnel-shaped lumen that is narrowest at the oral or ventral end. The musculature of the pharynx is similar in most details of its organization to that found in *Syndesmis* as described by Russo (1895). A thin superficial layer of vertical fibers overlies the well-defined muscles encircling the lumen of the pharynx. In addition to the circular and vertical muscles, radial fibers pass from the lumen to the peripheral surface of the pharynx. Nonmuscular cells with heavily staining reticular cytoplasm fill the spaces between the radial fibers (Fig. 8). Surrounding the pharynx is a sharply defined basement membrane to which are attached numerous short, radially arranged, protractor muscles that extend to the basement membrane of the ventral epidermis. The more oblique of these fibers serve also as dilators of the pharynx. Poorly developed retractors are attached to the equator of the pharynx and pass to the dorsal surface. Pharyngeal glands are present encircling the dorsal end of the pharynx. The

peripheral contours of these glands are lobular and a thin basement membrane separates them from the parenchyma. The cells which make up these glands have indistinct cell boundaries and dense cytoplasm containing numerous granules that stain darkly with hematoxylin. Cytoplasmic continuations of the cells extend ventrally and line the lumen of the pharynx (Fig. 8). Leading dorsad from the pharynx is a short oesophagus which passes through the pharyngeal glands and opens into the anterior end of the intestine.

The intestine lies along the mid-line under the dorsal epidermis and extends posterad from the level of the brain to about one-fourth of the distance from the posterior end of the body (Fig. 1). The width of the gut varies from 0.1 to 0.2 mm. at the anterior end and diminishes gradually posteriorly. Short diverticula extend laterally on each side. The epithelium of the intestine is made up of large irregularly shaped cells containing moderately granular cytoplasm. The basal end of most cells reaches the fibromuscular investing sheath of the intestine that separates it from the parenchyma. The lumen of the intestine can only be observed when ingested material is present; this condition is similar to that found in some alloecocoids. In an animal that has been feeding, food masses often lie in cavities that have lost all direct communication with the oesophagus (Fig. 1). Food vacuoles of varying sizes are generally present in the cells surrounding the ingested material and digestive cells were occasionally observed that had apparently migrated into the food masses by amoeboid movement.

Male reproductive system

The paired testes lie lateral to the mid-line in the anterior half of the body. They are approximately 0.5 mm. long and from 0.3 to 0.5 mm. wide. Each is made up of four to six vesicular lobes, the lumina of which are in direct communication with one another (Fig. 2). Separating the testes from the parenchyma is a fibrous sheath that penetrates and partially subdivides the lobes. The chambers so formed are filled with developing germ cells and tangled masses of mature spermatozoa (Fig. 3). Mature sperm are present in all lobes but are more numerous midway between the anterior and posterior ends of the testes near the wide openings of the sperm ducts. These ducts run mesially from the testes and enter the mid-ventral parenchyma, whereupon they diminish to about $10\ \mu$ in diameter and generally continue their course anterad, dorsolateral to the uterus (Figs. 1-3). A thin epithelium surrounded by loose fibromuscular elements makes up the walls of the sperm ducts. Near the origin of these ducts from the testes, glandular cells that probably possess some accessory function are found in the mid-ventral parenchyma adjacent to the ventral walls of the tubes (Fig. 2).

At varying distances posterior to the pharynx the sperm ducts unite mesially and enter the anterior end of a common sperm duct which at this point is somewhat enlarged to form a small spermiducal vesicle (Figs. 1-3). The slightly coiled common sperm duct continues posterad from the vesicle through the mid-ventral parenchyma. It gradually diminishes in diameter from $45\ \mu$ to $12\ \mu$. Its walls are composed of connective tissue cells surrounded by a sheath of circular, oblique, and longitudinal muscle fibers. The lumen of the tube is lined by a thin squamous epithelium that is separated from the theca by a thick basement membrane. Posteriorly, the common sperm duct unites with the enlarged base of the penis at

about one-third of the distance from the posterior end of the body (Figs. 1, 2, 4). The penis lies in a muscular sheath, the male antrum, which is a diverticulum of the genital antrum. Histologically this sheath is similar in most details of its structure to the common sperm duct; however, the lining epithelium of the male antrum is thicker, in some regions almost occluding the lumen, and a thick basement membrane is lacking (Fig. 5). The copulatory organ is a cuticular tubule that extends through the posterior third of the body and is about 3μ in thickness over most of its length. The lumen of the stylet does not exceed 2μ in diameter except at the anterior end of the penis which is enlarged to 12μ at its union with the posterior end of the common sperm duct (Figs. 1-3). The rim of the funnel-like base of the penis is thickened to form a collar; longitudinal muscles in the walls of the male antrum and common sperm duct attach to this collar and function as protractors and retractors of the penis.

Female reproductive system

The paired ovaries lie in the posterior third of the body. Each is made up of from five to ten lobes that branch dichotomously from common trunks arising near the anterior end of the seminal receptacle. The lobes of the ovaries are directed posterolaterad and are separated from the parenchyma by a very poorly developed theca. The branches are made up of dovetailed chains or rouleaux of compressed ova that are proliferated from primordial cells at the distal ends of the lobes (Fig. 2). Mature ova are approximately 75μ in diameter and vary in thickness from 20 to 60μ . The cytoplasm of immature eggs is at first homogeneous, but as development continues many small peripherally distributed granules appear that are probably stored nutrient materials. During the period of growth the nuclei of the ova increase from 7 to 25μ in diameter and the chromatin granules gradually lose their affinity for basic dyes. In mature ova only the spherical or oval nucleolus stains deeply with hematoxylin (Fig. 4).

A pair of greatly branched vitellaria lie anterior to the ovaries and fill most of the ventrolateral spaces in the middle third of the body (Figs. 1, 2). Many of the dorsoventral muscles of the parenchyma contribute fibers to the diffuse sheath that encloses these ducts. Primordial cells at the distal ends of the branches give rise to yolk cells. As the cells increase in size, the cytoplasm which at first is homogeneous, becomes filled with refractile granules that coalesce to form amber-colored droplets (Figs. 3, 4). From each side three or four collecting ducts packed with mature yolk cells pass posterad from the vitellaria and unite near the mid-line shortly before emptying into the anterior end of the ovovitelline duct (Fig. 2).

The seminal receptacle is somewhat oval and lies ventral to the intestine within the sheath that surrounds the gut. Its anterior extremity is about one-third of the distance from the posterior end of the body. The posterior part of this organ is thin walled and masses of mature spermatozoa are observable in its extensive lumen. Anteriorly the seminal receptacle opens with the paired ducts of the vitellaria and ovaries into the ovovitelline duct which arises ventrally in this region (Figs. 1, 2). The wall of the anterior third of the seminal receptacle is lined by large gland-like cells that restrict the lumen to a narrow channel 6 to 10μ wide which connects the posterior vesicular portion to the ovovitelline duct (Fig. 4).

The bursa seminalis lies dorsal to the vesicular portion of the seminal receptacle. It is enclosed in the same sheath that surrounds the seminal receptacle and the pos-

terior end of the intestine (Figs. 1, 2). The large lumen of the bursa seminalis is lined by an epithelial layer very similar to that lining the posterior part of the seminal receptacle. In every specimen examined spermatozoa were found in the bursa; frequently they were aggregated into roughly spindle-shaped masses in which degenerating sperm were observable (Figs. 5, 6). Arising ventrally, or in some cases laterally, from the wall of the posterior half of the bursa seminalis is the insemination canal, a fine cuticular tubule about 4μ in diameter connecting the lumina of the bursa seminalis and seminal receptacle. In close association with the insemination canal, a second cuticular tube of the same dimensions arises from the wall of the bursa seminalis and connects the bursa posteriorly to the bursal canal (Figs. 1, 2, 5, 6). Surrounding the ends of the ducts as they penetrate the lining epithelium of the bursa is a cuticular sheath, 7μ in diameter and 10μ long. The inner end of this sheath is involuted and fused to the ends of the two ducts (Fig. 6). To designate this composite cuticular structure made up of the insemination canal, the proximal end of the bursal canal and the sheath surrounding the ends of these ducts, the term, "bursal valve," is suggested.

The bursal canal (vagina) is a tubular structure about 0.1 mm. long and 20μ in diameter that arises as an anterodorsal continuation of the common genital antrum. Its wall is composed of an inner epithelial layer surrounded by a strong fibromuscular sheath. At the posterior end of the canal the epithelium possesses cilia-like projections characteristic of the lining of the common genital antrum. Anteriorly the lumen of the canal is reduced and the thin basement membrane underlying the epithelium becomes continuous with the cuticular wall of the tubule leading into the bursa seminalis.

A flattened muscular ovovitelline duct (ductus communis) arises ventrally near the anterior end of the seminal receptacle and receives the ducts of the ovaries and vitellaria. It passes posterad through the mid-ventral parenchyma to about the level of the posterior end of the bursa seminalis and here enters the anterior end of the female antrum (Figs. 1, 2, 5). The ovovitelline duct is approximately 35μ wide but is capable of considerable expansion to allow ova and yolk cells to pass into the uterus. Circular, oblique and longitudinal muscles are observable in contact with the thin basement membrane that underlies the lining epithelium; no fibrous sheath separates this duct from the cells of the mid-ventral parenchyma. Running parallel to the ovovitelline duct in the lateral parenchyma are paired accessory glands which enter the posterior part of the duct prior to its union with the female antrum (Figs. 1, 2, 5). Generally the cytoplasm of these gland cells stains evenly; however, in some cases the cells were observed to be filled with eosinophil granules.

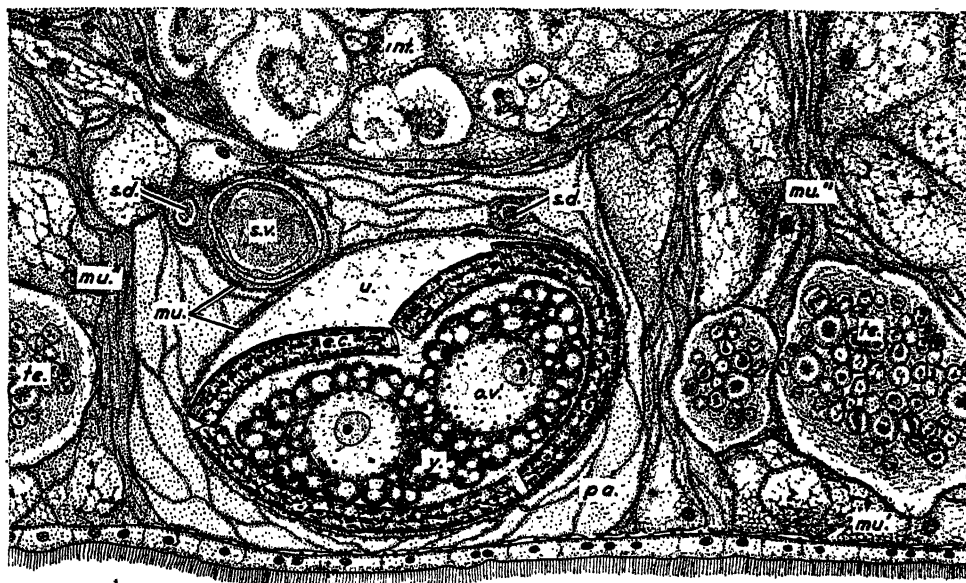
The uterus arises ventrally from the anterior end of the female antrum. It extends anterad almost to the pharynx through the mid-ventral parenchyma and

FIGURE 3. Transverse section through egg capsule and spermiducal vesicle ($\times 350$).

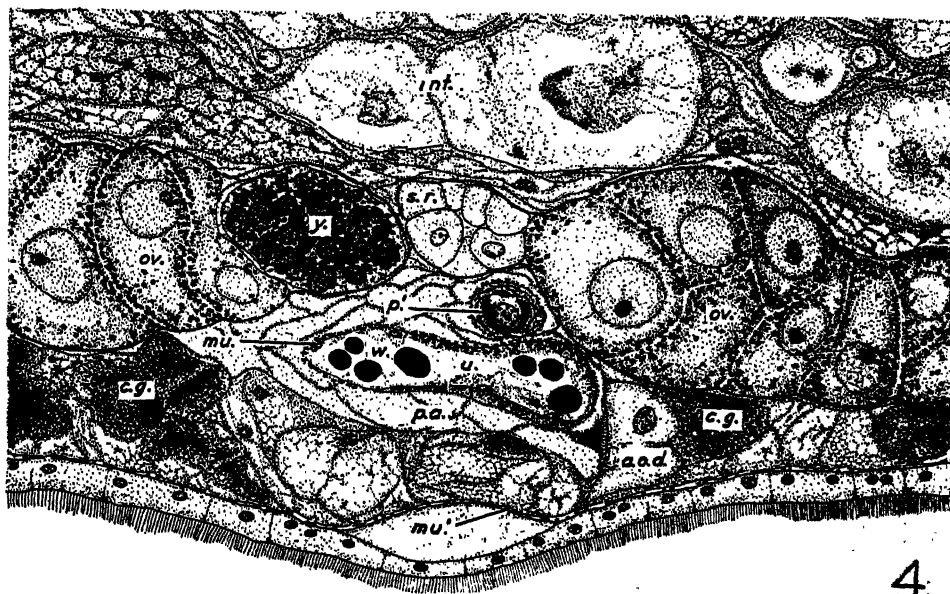
FIGURE 4. Transverse section through entrance of ovary into seminal receptacle ($\times 500$).

Abbreviations for Figures 3 and 4.

a.o.d.—accessory glands of ovovitelline duct, c.g.—cement glands, e.c.—egg capsule, int.—intestine, mu.—muscle sheath, mu'.—subepidermal muscles, mu''.—dorsoventral muscles of parenchyma, ov.—ovary, ov'.—ovum, p'.—base of penis, pa.—mid-ventral parenchyma, s.d.—sperm duct, s.r.—seminal receptacle, s.v.—spermiducal vesicle, te.—testis, u.—uterus, w.—whip of egg capsule, y.—yolk cells.



3



4

FIGURES 3-4.

through its entire course lies very close to the ventral surface of the body (Figs. 1, 2, 4). The anterior end of the uterus is enlarged and encloses an amber-colored, oval egg capsule containing numerous yolk cells and from one to five spherical eggs (Figs. 1, 2, 3). The egg capsule is cuticular and possesses a whip-like prolongation that extends posterad through the entire length of the uterus and female antrum. Over most of its length the whip is about 10μ thick. In the middle portion of the uterus the whip is often coiled back upon itself a number of times so that its total length may greatly exceed that of the uterus (Figs. 1, 2). The uterine wall is very similar in structure to the ovovitelline duct and is able to enlarge greatly to accommodate the egg capsule and the folded part of the egg whip (Figs. 3, 4).

The female antrum extends from the posterior ends of the uterus and ovovitelline duct to the common genital antrum (Figs. 1, 2). The walls are lined by columnar epithelial cells surrounded by a thin basement membrane and a muscular layer that is continuous with the fibers enclosing the uterus and ovovitelline duct. The lumen is about 12μ in diameter and the posterior end of the egg whip, when present, almost completely fills this space (Figs. 5, 7). The ventrolateral spaces of the posterior third of the body contain numerous unicellular cement glands. The cytoplasm of these cells is generally uniformly filled with small granules that have a strong affinity for hematoxylin. Throughout the entire length of the female antrum many ducts from these glands enter the lateral walls (Figs. 1, 2, 5, 7). The secretions of the cement glands are believed to be associated with the attachment of the egg capsules to the substrate when expelled. Living animals compressed under a cover glass were occasionally observed at low magnification to undergo a series of rapid contractions which resulted in the extrusion of the egg capsule and whip. However, nothing is known about the normal deposition and attachment of the capsules, nor are other details of the life cycle understood.

The common genital antrum lies at the posterior end of the body. It is an elongated tube lined by flattened cells that appear to have cilia about 20μ long which extend into the lumen (Figs. 1, 2, 7). A diffuse fibrous sheath separates this organ from the parenchyma. The common genital antrum receives the terminal ducts of both male and female reproductive systems: the bursal canal arises from it as a dorsal diverticulum; the male antrum enclosing the penis stylet is given off as a mesial evagination; and the female antrum enters it ventrally. The common genital pore opens on the ventral surface at the posterior end of the body. At this point

FIGURE 5. Transverse section through bursa seminalis and bursal valve ($\times 350$).

FIGURE 6. Bursal valve ($\times 1,050$).

FIGURE 7. Transverse section through the entrance of female antrum and male antrum into the common genital antrum ($\times 350$).

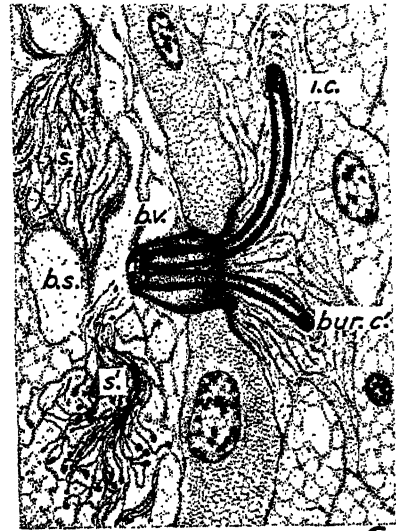
FIGURE 8. Transverse section through pharynx ($\times 200$).

Abbreviations for Figures 5 through 8.

a.o.d.—accessory glands of ovovitelline duct, a.o.d'.—ducts of accessory glands of ovovitelline duct, b.c.—buccal cavity, bur. c.—bursal canal, bur. c'.—cuticular end of bursal canal, b.s.—bursa seminalis, b.v.—bursal valve, cil.—cilia, c.g.—cement glands, d.c.g.—ducts of cement glands, f.a.—female antrum, g.a.—common genital antrum, int.—intestine, i.c.—insemination canal, l. int.—lumen of intestine, m.a.—male antrum, mu.—muscle sheath, mu'.—subepidermal muscles, mu''.—pharyngeal protractor muscles, n.—nerves, oe.—oesophagus, o.d.—ovovitelline duct, pa.—mid-ventral parenchyma, p.—penis, ph.—pharynx, ph. g.—pharyngeal glands, s.—spermatozoa, s'.—degenerating spermatozoa, u.—uterus, w.—whip of egg capsule.



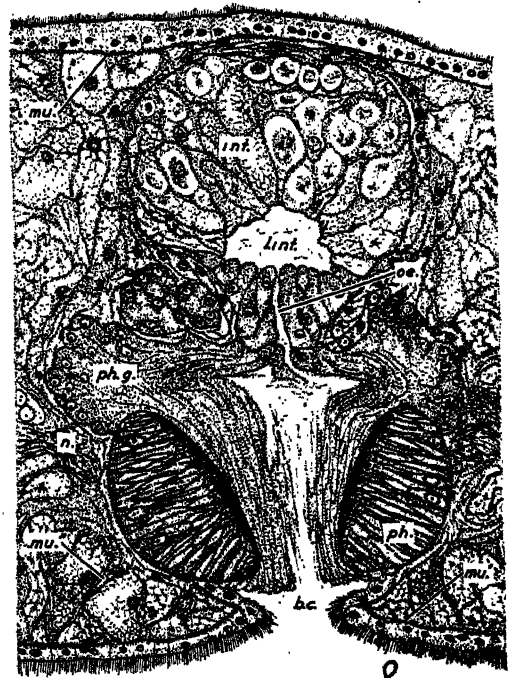
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FIGURES 5-8.

the ciliated ventral epithelium is invaginated and forms a short bulb-like canal which meets an outpocketing of the common genital antrum. Sphincters encircle both ends of this canal and regulate the size of the pore.

DISCUSSION

Comparison of genera

Although the parasite described here is similar in many respects to all genera in the family Umagillidae, there are certain structural characteristics that do not correspond to those of any previously reported genus of this family. Therefore, it is considered necessary to establish a new genus to be designated by the name *Syndisyrix*. This name is intended to describe the complex bursal valve which is not present in any other genus of the family. The specific name, *Syn. franciscanus*, is given to designate the host, *Strongylocentrotus franciscanus*, in which it was first found.

For the sake of uniformity in the following comparison of genera of the family Umagillidae, the morphological nomenclature used by the various authors in their original descriptions of genera and species has been altered to conform with the terminology employed in the preceding analysis of *Syndisyrix*.

In addition to the fact that both *Syndisyrix* and *Syndesmis* are found in the intestine of echinoids, the morphological characteristics of *Syndisyrix* indicate a closer relationship to *Syndesmis* than to the other genera of the family. The location and appearance of important organs, *vis.*, muscular pharynx, lobed testes, small spermiducal vesicle, muscular common sperm duct, ramified vitellaria, dichotomously branched ovaries, elongated uterus and egg capsule with whip, are very similar in *Syndesmis* and *Syndisyrix* and strongly suggest a close relationship between these two genera. *Syndisyrix* differs from *Syndesmis* chiefly in the structure and relationships of the bursa seminalis and seminal receptacle. In *Syndesmis* a single vesicle is present for the reception of sperm and cuticular structures such as the parts which make up the bursal valve of *Syndisyrix* are lacking. In addition to these differences, the structure of the penis is markedly dissimilar in these two forms. The penis of *Syndisyrix* is a cuticular hollow stylet attached only at the base, whereas the copulatory organ of *Syndesmis* is a muscular eversible tube with a cuticular lining (Russo, 1895; Fig. 16).

Structures corresponding to the cuticular canals in the bursal valve of *Syndisyrix* are found in *Anoplodiera voluta*, *Wahlia macrostylifera*, and *Desmote vorax*. In *A. voluta* the relationships of the two cuticular canals to the bursa seminalis, as described by Westblad (1930), are very similar to the arrangement of these structures in *Syndisyrix*. However, the cuticular sheath that surrounds the entrance of these ducts into the bursa is lacking in *A. voluta*. There do not appear to be grounds for concluding that *Syndisyrix* and *Anoplodiera* are closely related since the appearance and location of the testes and vitellaria, the presence of a single ovary, and the absence of a female antrum connecting the ovovitelline duct and uterus to the common genital antrum in *A. voluta* differ strikingly from the arrangement found in *Syndisyrix*.

In *W. macrostylifera*, described by Westblad (1930), and *D. vorax*, according to Beklemishev (1916), the proximal end of the bursal canal is cuticular but an insemination canal is lacking. In other respects *W. macrostylifera* differs from

Syndisyrynix chiefly in regard to the morphology of the male reproductive system. The penis stylet is greatly elongated, and paired sperm ducts arising from compact testes unite and communicate by means of a single duct with the large spermiducal vesicle situated anterior to the pharynx. Many points of difference are likewise found by comparing the morphology of *Syndisyrynix* and *Desmote*. The most evident of these are the bipartite gut and the presence of two genital pores, the anterior pore by which the uterus opens to the exterior and the posterior pore which serves for copulation in *D. vorax*.

The other genera of the family lack cuticular parts in the copulatory complex comparable to those in the bursal valve of *Syndisyrynix* and to a greater or less degree exhibit dissimilarities in the location, distribution, number, arrangement and relationships of organs in the body. In these genera the most conspicuous differences with respect to *Syndisyrynix* are: the single ovary and absence of a cuticular copulatory stylet in the genus *Anoplodium*; the unbranched ovaries and double-walled cuticular penis stylet in the genus *Umagilla*; the absence of a cuticular penis and the general arrangement of testes and vitellaria in the genus *Xcnometra*; and the single testis in the genus *Collastoma*. A manuscript is in preparation which will deal at greater length with the structural relationships of these forms.

Bursal valve

There is a superficial similarity between the bursal valve of *Syndisyrynix* and the cuticular nozzle-like mouthpieces of acoels. In the acoel, *Amphichoerus*, described by Graff (1891), and many allied forms, one end of the mouthpiece is generally connected to a vesicular sac or bursa filled with sperm; the other end is directed toward the ovary. L. H. Hyman (1937) points out that the function of these mouthpieces is apparently to direct sperm toward the ova to help insure fertilization. This function can hardly be ascribed to the insemination canals of *Anoplodiera* and *Syndisyrynix* which conduct sperm from the bursa seminalis to the seminal receptacle and not directly to the ova; nor does it seem probable that the insemination canals of *Umagillidae* are homologous to these mouthpieces. Noncuticular ducts connect the bursa seminalis to the seminal receptacle and bursal canal in most genera of *Umagillidae*, which suggests that cuticular structures are probably of relatively recent rather than primitive origin. In an analysis of the existing genera, Wahl (1910b) presents evidence which leads him to conclude that *Umagilla* is the most primitive and least modified genus of the family. If one accepts this view, it lends support to the opinion expressed above, inasmuch as *Umagilla* lacks any cuticular structures that might be considered homologous to the bursal valve. It is possible that the absence of cuticular parts in some of the species is due to a greater degree of simplification associated with a parasitic existence. However, there is no direct evidence for this supposition, since in the most closely related free-living families, *Graffillidae* and *Dalyelliidae*, cuticular structures such as these are not found. This suggests that these tubules have arisen independently, and until additional information is available, the insemination canals and bursal canals of *Umagillidae* should not be considered as mouthpieces in a true sense.

Although copulation has not been observed in *Syndisyrynix*, it is believed that the sperm of one animal are injected by means of the protrusible penis into the bursal canal of another. Before fertilization can take place, sperm must migrate from the

bursal canal through its narrow proximal end into the bursa seminalis, there remaining until able to find their way through the insemination canal into the seminal receptacle. Evidently many sperm are unable to accomplish this migration and degenerate in the lumen of the bursa seminalis. Sperm that do reach the seminal receptacle must then pass through the constricted anterior part of this organ to fertilize the mature ova that enter the ovovitelline duct at the anterior end of the seminal receptacle.

It is difficult to explain any selective advantage for the presence of the fine canals that make up the bursal valve of *Syndisyrix*. It was thought at first to be a mechanism for the prevention of polyspermy. However, this explanation is negated by the presence of large masses of spermatozoa in the seminal receptacle. The simplest explanation for the presence of these ducts is that they act as valves which regulate the number of spermatozoa entering the bursa seminalis and seminal receptacle. If this interpretation is correct, it is probable that the function of the bursal valve is to insure a necessary aging of the sperm in the bursa before fertilization. The cuticular walls are necessary to prevent the collapse of these narrow tubes. It is evident that the bursal valve restricts the free passage of sperm from the bursa seminalis and therefore as the result of a single copulation, a continuous supply of sperm may be maintained over a long period of time.

SUMMARY

After completing a histological study of an endoparasitic rhabdocoel from the Pacific Coast sea urchin, *Strongylocentrotus franciscanus*, the following conclusions have been reached:

1. This parasite belongs to the rhabdocoel family Umagillidae but differs in certain characteristics from the eight known genera of the family.
2. The distinguishing characteristics are a single intestine, paired and lobed ovaries and testes, a tubular single-walled cuticular penis stylet, and cuticular ducts connecting the bursa seminalis to the bursal canal and seminal receptacle.
3. A characteristic structure typical of this parasite and not present in other genera of the family is the bursal valve composed of two cuticular tubes, the insemination canal and proximal end of the bursal canal, which enter the bursa seminalis through a cuticular cup-like sheath.
4. The parasite here described is given the name *Syndisyrix franciscanus*, gen. et sp. nov.

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A QUANTITATIVE STUDY OF THE RELATIONSHIP BETWEEN THE ACTIVITY AND OXYGEN CONSUMPTION OF THE GOLDFISH, AND ITS APPLICATION TO THE MEASUREMENT OF RESPIRATORY METABOLISM IN FISHES

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INTRODUCTION

The fact that fish consume more oxygen when active than when quiescent has been observed by many investigators (Krogh, 1916; Bowen, 1932; Clausen, 1933, 1936; Wells, 1935; Schlaifer, 1938; Smith and Matthews, 1942), but apparently no attempt has been made to determine the exact relationship between oxygen consumption and activity in fishes. It is the purpose of this paper to present data which are believed to provide an objective and quantitative basis for the relationship between activity and oxygen consumption in the goldfish, and to describe a method for making the necessary measurements. The method is based on the use of a recording activity detector (Spoor, 1941) combined with a continuous flow system for measuring oxygen consumption.

The lack of definite information on the activity of fish under experimental conditions has been one of the chief sources of difficulty in work on the respiratory metabolism of fishes, and attention has been called to the need for an experimental method which would make it possible to distinguish between "standard metabolism" and the increased metabolism due to muscular movements (Wells, 1935). In view of the fact that the oxygen consumption is affected by changes in the basal metabolic rate as well as by changes in activity, the importance of such a method is apparent. The method employed in the present work seems to meet this need, inasmuch as the state of activity is recorded continuously and periods of inactivity can be selected for measuring basal oxygen consumption.

Szymanski (1914) and Spencer (1939), using other types of activity detectors, have reported that goldfish show considerable individual variation in activity and that the activity pattern is affected by light. Spencer (1939) also found activity to be influenced by food. Knowledge of the behavior of the fish under the experimental conditions is of importance in the collection of data on oxygen consumption in the method to be described, as well as in the interpretation of these data. For this reason further observations on the patterns and rates of activity and on the effects of food, light and disturbances are included in the present paper.

THE ACTIVITY OF THE GOLDFISH UNDER EXPERIMENTAL CONDITIONS

Method

Several dozen goldfish (*Carassius auratus*) ranging between 24 and 96 grams in weight were selected at random from a stock obtained from a local goldfish farm

and studied individually in experimental chambers, each of which was equipped with a recording activity detector. The experimental chambers were set up in a ground floor aquarium room which was seldom entered except for the purposes of this study, so that the fish could be left for long periods with relatively little disturbance. The recording apparatus was kept in another room. Records of the activity of each fish were started shortly after its introduction into a chamber and continued for periods ranging from a few days to many months in length, during which the patterns and rates of activity and the effects of food, light and disturbances upon them were studied. With a few exceptions, oxygen consumption was not measured in this series of observations.

The experimental chamber (Fig. 1) consisted of a one-gallon brown glazed

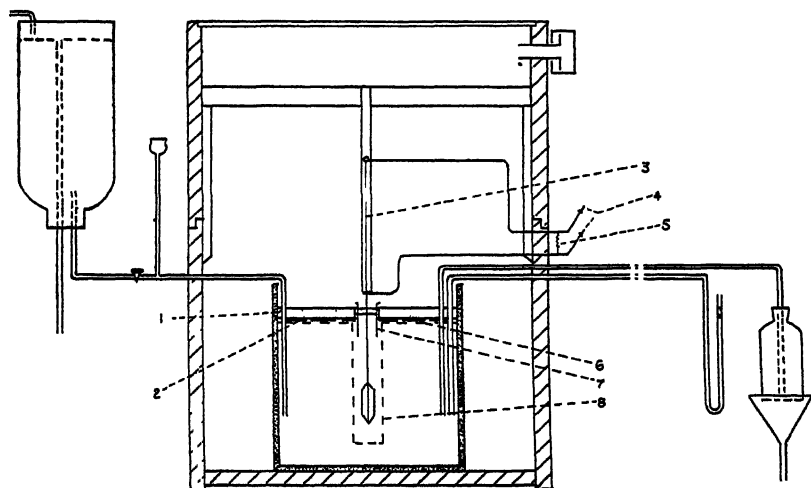


FIGURE 1. Diagram of apparatus for measuring oxygen consumption and activity. (1) paraffin oil (this was omitted when activity alone was being measured), (2) glass plates, (3) No. 44 copper wire, (4) sensitive relay, (5) resistor, (6) wire screen, (7) glass tube, (8) wire frame protecting paddle. Explanation in text.

crook fitted with a galvanized iron wire screen of $\frac{1}{2}$ inch mesh to prevent the fish from reaching the surface of the water. A glass tube about 3 cm. in diameter was fitted into an opening in the center of the screen so that it extended 3.5 cm. above and 3 cm. below the screen; its purpose will be considered in a later section. The surface of the water stood about 3 mm. above the screen, the total volume to this level being 2,600 cc. Water entered the chamber from a constant level reservoir through 8 mm. glass tubing and left by way of a siphon of 8 mm. glass tubing which dipped into a constant level drain, the rate of flow (between 70 and 100 cc. a minute) being regulated by means of a glass stopcock in the inlet. The intake of the siphon was placed about 5 cm. above the bottom of the chamber, so that feces and other debris that fell to the bottom did not enter the siphon until they had been broken into small pieces in the course of their passage upward to the intake. The chamber was practically self cleaning under these conditions, the flow of the water and the movements of the fish being sufficient to move debris into the siphon. The fish

could therefore be maintained in the chamber for months without cleaning. A thistle tube entering the inlet provided for the introduction of food, being closed off at all other times. The water supply consisted of tap water passed through an activated charcoal filter, brought to the desired temperature and aerated until it approached equilibrium with the atmosphere. Most of the observations were made at temperatures between 20 and 24° C. The fish seldom extracted more than one-third of the oxygen from the water at the rates of flow employed, and they usually took less than this. In view of the findings of Crozier and Stier (1925), Toryu (1927), and Schlaifer (1938), it seems unlikely that behavior was influenced by the oxygen tension of the water.

The chamber was enclosed in a wooden case to minimize disturbances and to make it possible to control the light. The top of the case was fitted with a pane of glass for natural illumination, and with a wooden cover when either complete darkness or constant light was desired. A ventilator in the side of the case, with baffles to prevent light from entering, permitted some circulation of the air. The water inlet, outlet siphon, and a tube leading to a U tube indicating the water level in the chamber passed through the wall; a coat of black paint over each tube prevented light from entering the chamber through these openings.

The detector consisted of a light-weight aluminum paddle suspended in the water in the experimental chamber by a fine copper wire in such a way that a silver rod at the top of the paddle shaft passed through a small hole in a fixed silver plate. Water currents set up by the movements of the fish moved the paddle, causing the rod to make and break contact with the sides of the hole and thus to activate a sensitive relay. This relay operated the recording apparatus. The blade of the paddle consisted of aluminum foil (5 cm. long and 2.5 cm. wide) with the corners bent in at right angles so that the water currents struck a flat surface regardless of their points of origin. The shaft (10 cm. long) consisted of no. 22 aluminum wire cemented to the blade and imbedded at its upper end (7 cm. above the blade) in a bakelite insulating rod (2 cm. long and 0.2 cm. in diameter) in the upper end of which the silver rod (1 cm. long and about 0.04 cm. in diameter) was imbedded. This silver rod was soldered to a 14.5 cm. length of no. 44 enameled copper wire held in an insulated binding post attached to a wooden supporting shaft. A wooden bracket rising from the case supported this shaft in a vertical position so that the paddle hung in the water through the glass tube in the center of the screen. A cylindrical frame of galvanized iron wire protected the paddle from contact with the fish. A small lead weight (about 0.1 gm.) clamped to the paddle shaft below the bakelite helped to bring the paddle back to the resting position after displacement by the water currents. The silver plate (about 0.5 cm. square) was attached to the supporting shaft and held in a horizontal position about 6 cm. above the screen. The hole in the plate was between 0.08 and 0.1 cm. in diameter. The current to operate the sensitive relay was supplied by a 6 volt storage battery; the coil of the relay had a resistance of 1,000 ohms. A 5,000 ohm resistor across the detector contacts prevented sparking and welding without causing an observable reduction in the sensitivity of the detector. The plate was kept warm by means of a small insulated heating coil in order to prevent water from condensing upon it from the humid atmosphere above the chamber.

The sensitivity of the detector could be controlled somewhat by adjusting the position of the silver rod with respect to the sides of the hole in the plate. The nor-

mal movements of the operculum and the position-maintaining fin movements of a quiescent 25- to 30-gram goldfish were usually sufficient to move the paddle slightly, and when the rod was close to the plate these movements were recorded. For the observations to be described, however, the rod was centered in the hole so that the ordinary respiratory movements did not move the paddle enough to make contact, these movements being considered as among the basal functions of the fish. Vigorous respiratory movements and any movement that resulted in a change in the position of the fish moved the paddle enough to make and break contact, slow swimming movements causing few, and vigorous activity causing many impulses to be recorded. At the flow rates used in these experiments the flow of water through the chamber did not move the paddle.

The sensitive relay activated a counter which in turn caused signal magnets to record every tenth and hundredth impulse on a long paper kymograph moving about 30 mm. an hour. The frequency of the impulses was such (ranging up to 6,000 an hour) that they usually could not be counted when recorded individually. The counter was capable of following and recording at least 10 impulses a second. Time was recorded in hours beneath the activity record.

Patterns and rates of activity

In agreement with the results of Szymanski (1914) and Spencer (1939), the goldfish used in this study proved to be quite variable in their patterns and rates of activity, even when they were maintained under almost identical conditions of light, feeding, temperature, water supply and disturbance. Three general types of behavior appeared when the fish were kept under natural conditions of light: (1) arrhythmic activity, in which no relation to day or night could be detected; (2) rhythmic activity, in which the fish were active by day and quiescent at night; (3) rhythmic activity, in which they were quiescent by day and active at night.

Fish showing the first, arrhythmic, type of behavior were extremely variable. A few were vigorously active day and night for periods as long as ten days, others were moderately active throughout the 24-hour period for weeks at a time, and still others remained practically inert for similar periods. Some of these arrhythmic fish, particularly those in the last group, showed irregular bursts of activity now and then, with no apparent relation to the time of day, feeding, or disturbance.

An example of the second type of behavior, diurnal activity and nocturnal quiescence, is shown in Figure 2, which is based on the number of impulses recorded by a 35-gram male goldfish during each hour between 1 P.M. January 11 and 1 P.M. January 13, 1946. The fish was fed at 11:05 A.M. on January 12, otherwise the room was not entered between 2:45 P.M. January 11 and 7:15 P.M. January 13. Aside from the feeding, the effects of which are discussed below, light was the only known variable, the temperature, rate of flow and aeration of the water being the same at the end as at the beginning of the period. Most of the fish showing rhythmic changes in activity followed patterns of this type, although the active phase varied considerably, sometimes being interrupted by several hours of quiescence during the day, sometimes beginning later in the day, and occasionally continuing well into the night. The most constant period of quiescence occurred between midnight and 4 A.M., which is in agreement with Spencer's (1939) observations.

The third type of behavior, diurnal quiescence and nocturnal activity, was found less frequently than the second, although it was not uncommon. An example is

shown in Figure 3, which is based on records of the activity of a 32-gram male goldfish between 1 P.M. July 3 and 1 P.M. July 5, 1945. The room was not entered between 4 P.M. July 3 and 8 A.M. July 5, and aside from the daily changes in light the environmental conditions apparently remained constant throughout the period.

The patterns of rhythmic fish did not seem to be fixed, however, even when the environmental conditions remained unchanged. After several weeks of rhythmic behavior the fish frequently became arrhythmic for several weeks or months, occasionally becoming rhythmic again in the course of extended periods of observation. This suggests that those fish which did not show daily activity rhythms under the

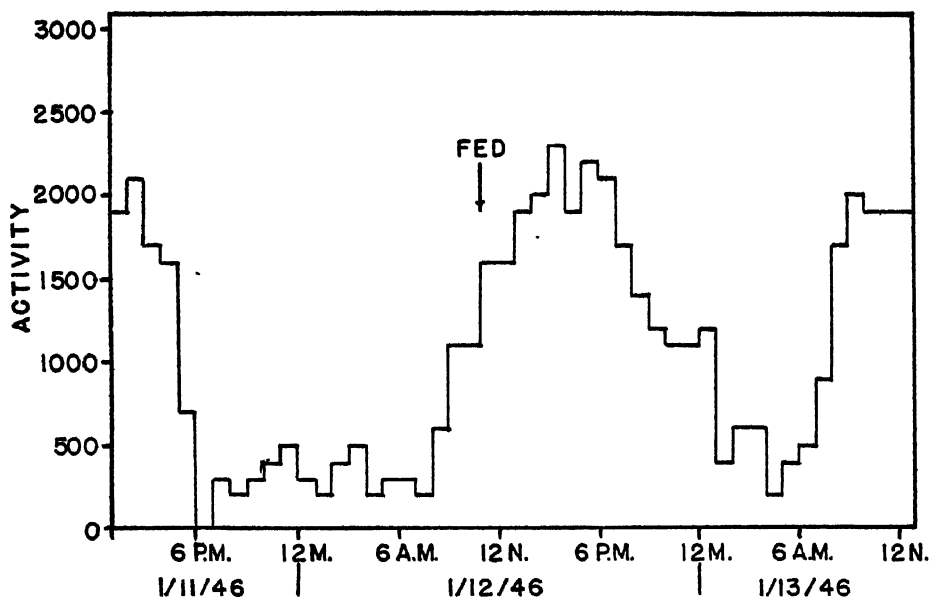


FIGURE 2. Activity pattern of 35-gram male goldfish between 1 P.M. January 11 and 1 P.M. January 13, 1946. Activity is expressed as number of impulses recorded each hour. Temperature 21.5° C. Fed at 11:05 A.M. January 12.

experimental conditions may have done so eventually had they been studied for longer periods, and that by chance the observations were made during arrhythmic periods.

Activity and food

The fish were fed rolled oats, commercial fish foods, shredded shrimp, ground liver or chopped earthworms about three times a week, usually 0.5 to 1 gram at each feeding. The effects of daily feeding, larger amounts of food and starvation were also studied. Under the conditions of the experiments the type of food given had no consistent effects upon activity, but the quantity of food had pronounced effects, particularly on the total amount of activity. A well fed fish was usually sufficiently active that the number of impulses recorded in the course of a 24-hour period averaged between 500 and 1,500 an hour, and averages in excess of 2,500 impulses an

hour were not uncommon. Starvation caused this rate to decrease markedly, sometimes to fewer than 100 impulses an hour, although as a rule the lowest rates did not appear until the fish had been starved for a week or so. No fish was observed to become completely inactive for periods of more than an hour or two, however, even when starved for two weeks. The effects of feeding after a period of starvation were striking, activity increasing to normal "well fed" rates within a few minutes. Doubtless the swimming movements associated with feeding accounted for some of the activity recorded following the introduction of food, but it seems that the nutritional state also affected the amount of activity. Food given in amounts of one

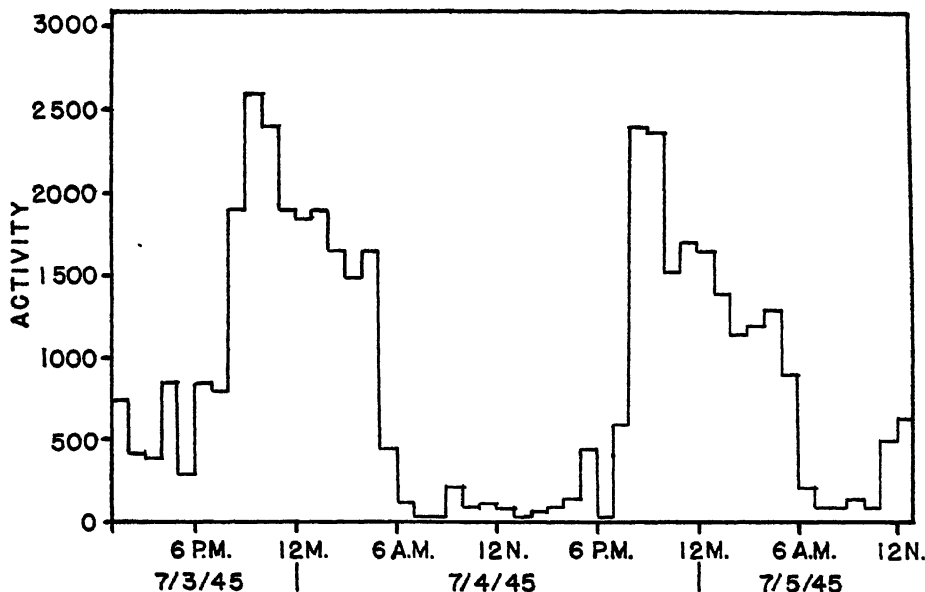


FIGURE 3. Activity pattern of 32-gram male goldfish between 1 P.M. July 3 and 1 P.M. July 5, 1945. Units as in Figure 2. Temperature 23.5° C.

gram or less was usually consumed within three to six hours, but the fish remained active (in accordance with their activity patterns) for from several days to a week after they had been fed. Similarly, Spencer (1939) found the goldfish to maintain a high rate of activity for several hours after feeding, although in his experiments the food was usually consumed within 15 minutes or less.

The effects of feeding upon activity rhythms were not studied in detail, but the available data bearing on this question indicate that although the rhythms appearing under natural conditions of light were frequently modified by the quantity of food and the time of feeding, they were not causally related to food. Feeding modified the activity patterns of some fish for part or all of the subsequent 24-hour period, usually by prolonging the active phase. A response of this type may be seen in Figure 2. The fish was fed 0.5 gm. of rolled oats at 11:05 A.M. on January 12 (the previous feeding being on January 9); it will be noted that the activity level remained relatively high for a much longer period on the night of January 12 than on

the preceding night. On the other hand some fish showed no change in activity in response to feeding, provided of course that they had not been starved. Variations in the quantity of food and in the time and frequency of feeding did not seem to have permanent effects on the activity rhythms, and feeding at the same time each day did not cause arrhythmic fish to become rhythmic.

Activity and light

The goldfish did not seem to be much affected by changes in light intensity while they were not following daily activity rhythms, but they were usually quite responsive to light during their periods of rhythmic behavior. In fact, when the fish were well fed and undisturbed the activity rhythms seemed to be closely related to the daily changes in natural light, as Szymanski (1914) has reported previously. This view is supported by several observations in addition to the fact that the active and quiescent phases of the cycles usually coincided with day and night. Periods of nocturnal activity and diurnal quiescence were shown by the 32-gram male goldfish mentioned above in July and December of 1945. Although the water temperature and other factors except light were the same during both periods, the nocturnal phase of activity usually began earlier in the evening (between 5 and 6 P.M.) and ended later in the morning (between 7 and 8 A.M.) in December than in July, when it usually began between 7:30 and 8:30 P.M. and ended between 5 and 6 A.M. This suggests of course that the nocturnal phase of activity was limited by the setting and rising of the sun. This fish also responded readily to experimental changes in light intensity, particularly during the day, when darkening the chamber caused its activity to increase to levels usually reached only at night. Records were also obtained in which diurnally active and nocturnally quiescent fish remained active on nights when bright moonlight entered the room in which they were kept. Spencer (1939) found that the regular diurnal rhythm of the goldfish could be obliterated by covering the tank by day and lighting artificially at night. This procedure was accompanied by night feeding, however, so that the change in activity may not be attributed solely to the reversed lighting.

On the basis of these observations attempts were made to maintain goldfish at definite rates of activity by exposing them to continuous dim light and to continuous darkness for periods lasting as long as three weeks, but without success. The fish did not maintain constant rates of activity under either condition, but continued to alternate periods of increased activity with periods of relative quiescence. In order to maintain a low rate of activity it was necessary to starve the fish for about a week, the relationship between nutritional state and amount of activity being similar to that described in the preceding section.

Activity and disturbance

The goldfish proved to be extremely sensitive to disturbances. Noise, slight changes in the water level, sudden lights, the mere presence of the observer in the room, or such minor disturbances as the quiet opening and closing of the door to the room usually caused a change in the rate of activity. Fish that had been active before the disturbance almost invariably became less active, sometimes practically motionless, while quiescent fish frequently, although less consistently, became active when disturbed. Whichever the response, the original state of activity was

usually resumed within a few minutes after the disturbance had ceased. The degree of response seemed to be related to the amount of disturbance, for when the observer moved slowly and quietly the change in activity was usually less pronounced, and recovery more rapid, than after ordinary passage through the room or adjustment of the apparatus. The effects of disturbances upon the activity of an otherwise quiescent fish may be seen in Figure 3. The room was entered several times in the course of the afternoon of July 3 and on July 5, although the experimental chamber was not approached and the fish could not see the cause of the disturbance. It is obvious that the rates of activity were higher than at corresponding hours on July 4, when the room was not entered. Such sensitivity has been observed in other species of fish by Clausen (1934), who found that a shadow passing over the aquarium caused increases in the body temperatures of perch and members of the sunfish group.

THE RELATIONSHIP BETWEEN ACTIVITY AND OXYGEN CONSUMPTION

Method

The activity and corresponding oxygen consumption of individual goldfish were measured in observation periods ranging in length from 11 to 210 minutes. Activity was measured in terms of the number of impulses recorded in a given period, and the amount of oxygen consumed by the fish in that period was determined by means of a continuous flow system. A control chamber similar to the experimental and housed in the same case was supplied with a continuous stream of water from the reservoir supplying the fish. The water in each chamber was covered with a layer of heavy paraffin oil 2.5 cm. thick to retard the diffusion of oxygen from the air, and a sample of the effluent from each chamber was analyzed for oxygen by the Winkler method at the beginning and end of each period. The samples were collected in narrow necked glass stoppered bottles of about 270 cc. capacity arranged to serve as constant level drains (Fig. 1). Each line was arranged so that the water passed through the outlet siphon to the bottom of the sampling bottle and overflowed into a funnel so that it could be collected for flow rate determinations. Although the rates of flow ranged from 70 to 100 cc. a minute in the course of the study, the rate for any one day's series of samples was held practically constant. Due care was taken to prevent the diffusion of oxygen into the samples and to obtain representative samples from experimental and control lines. Samples that were contaminated by particulate matter were discarded. The permanganate modification was used in most of the analyses, but was omitted during some of the shorter periods. The results obtained with and without the modification were quite similar, however, which was not unexpected in view of the fact that from four to six liters of water passed over the fish each hour.

The volume of water flowing through the system in the course of an observation period being known, together with the oxygen content of the water leaving the control and experimental chambers at the beginning and end of that period, the oxygen consumed by the fish could be calculated. The calculations took into account the change in the amount of oxygen in the constant volume of water in the chamber. The volume of water displaced by the fish was too small to affect the calculations.

The samples were collected with the foregoing observations on activity patterns

and modifying factors in mind, the periods being timed to yield data at the activity rates desired, and the method of sampling being modified as necessary to minimize disturbance of the fish. In the latter connection the outlet tubes were lengthened so that samples were collected about 10 feet away from the chambers, and the room was not entered except for sampling and rate of flow determinations. Precautions were taken to prevent changes in the water level in the experimental chamber as there were indications that small changes in the level stimulated the fish. These precautions were necessary also because the volume of water in the chamber, as well as that flowing through it, entered into the calculations of oxygen consumption. Samples were discarded if subsequent examinations of the activity records showed that they had been collected while the fish was undergoing marked changes in activity as a result of disturbance or in accordance with an activity rhythm. This was necessary because although the activity record was instantaneous the change in the oxygen concentration of the samples tended to lag somewhat behind that in the chamber, the sample drawn at any instant representing the average of the water flowing into the bottle in the few minutes preceding its removal. The temperature of the water was recorded for each observation period in order to avoid discrepancies attributable to the effect of temperature on metabolic rate (Ege and Krogh, 1914).

Owing to its viscosity and the accumulation of emulsified oil and water at the oil-water interface, the layer of oil interfered with the movements of the paddle shaft. Its thickness was therefore reduced to 1 cm. within the central glass tube, thus permitting the paddle to move about as freely as with a water surface. This tube extended below the interface far enough to prevent the emulsion from accumulating around the paddle shaft. The oil within the tube had to be changed now and then, however, to remove the small amount of debris that entered it from beneath. The detector contacts and bakelite rod were cleaned every few days as a precaution against their becoming coated with oil, which seemed to spread slowly up the paddle shaft.

It was established by appropriate tests that the layer of paraffin oil was effective in preventing the diffusion of significant amounts of oxygen into the water from the atmosphere. In no test did the apparent leakage exceed the limits of error of the Winkler method itself (Allee and Oesting, 1934), and it was usually considerably less. The average apparent rate of change for the contents of the experimental chamber was 0.0015 cc. of oxygen a minute, which was so much smaller than the rate at which the fish consumed oxygen that even had the apparent change been real it would have had but little effect on the results. It should be mentioned in this connection that the oil layer was disturbed relatively little by the movements of the fish, inasmuch as the wire screen kept the fish out of the oil and glass plates resting on this screen lessened the churning effects of the water beneath it.

Results

Three goldfish were studied at several temperatures in a total of 104 observation periods. As the results on all three were much alike, data on but one of the fish, a 32-gram male on which over two-thirds of the measurements were made, are presented here.

The relationship between activity and oxygen consumption at temperatures between 23 and 25° C. is shown in Figure 4, in which oxygen consumption in cubic

centimeters per minute is plotted against activity in impulses per minute. Fifty-nine observation periods are represented, each point corresponding to one period. The line merely indicates the trend, and has not been fitted to the data mathematically. As was to be expected, oxygen consumption and activity proved to be closely related, the relationship apparently being linear above the basal level of oxygen consumption. Although the values for oxygen consumption at any one rate of activity are seen to vary somewhat, the trend is clear cut: at high rates of activity the rate of oxygen

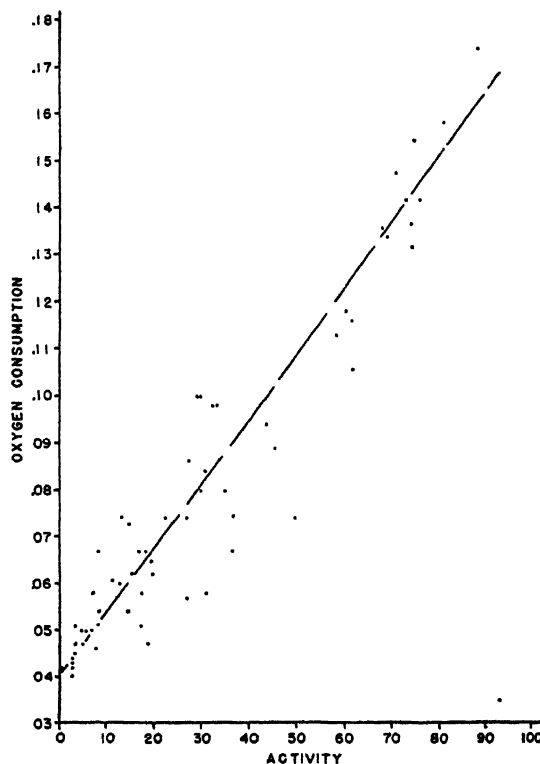


FIGURE 4. Activity and oxygen consumption of 32-gram male goldfish. Activity in impulses/minute; oxygen consumption in cubic centimeters/minute. Temperature 23 to 25° C.

consumption is correspondingly high; at low activity rates less oxygen is consumed. The discrepancies that do occur may well have been due to errors in measurement, rather than to a lack of correspondence between activity and oxygen consumption. In this connection the data on oxygen consumption follow those on activity quite closely when the comparison is restricted to one day's series of measurements, thus without discrepancies attributable to slight differences in the adjustment of the detector contacts. Such a series is shown in Figure 5, which is based on data obtained with the same fish in a series of thirteen consecutive 15- to 25-minute periods at 22° C.

According to the slope of the data shown in Figure 4, the basal oxygen con-

sumption of this fish was in the vicinity of 0.040 cc. a minute, or 0.075 cc. per gram per hour.

DISCUSSION

The results of the present study have a bearing on the collection and interpretation of data on the respiratory metabolism of fishes, and in the light of these results the method described seems to offer a number of advantages not found in previous methods which have been employed for this purpose.

The advantages of the continuous flow method for measuring respiratory metabolism in fishes have been discussed by Keys (1930) and need not be reviewed here. In view of the relationship between oxygen consumption and activity, however, the

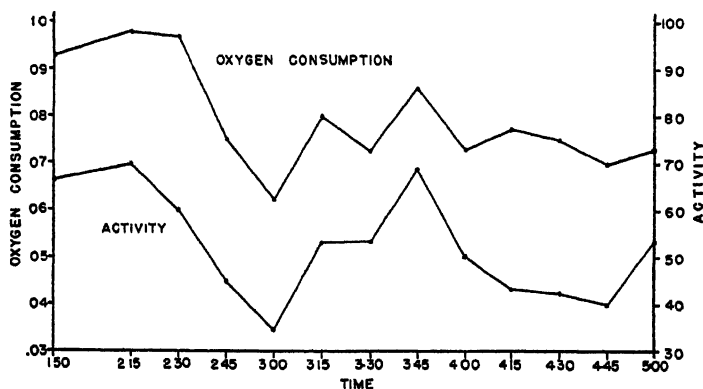


FIGURE 5. Activity and oxygen consumption of 32-gram male goldfish in each of thirteen consecutive observation periods between 1:35 P.M. and 5 P.M. November 13, 1945. Units as in Figure 4. Each point on the upper line represents the average rate of oxygen consumption for the 15- or 25-minute period preceding it. Each point on the lower line represents the average rate of activity for the corresponding period.

observations on the effects of disturbances may be applied to the use of this method, inasmuch as the process of sampling may disturb the fish. Should a change in the rate of activity (and consequently of oxygen consumption) occur at the time of sampling, the sample would not be representative of the volume of water and unit of time to which it is related in the calculations. The resulting error could be of considerable importance, particularly in investigations in which the samples consisted of water flowing directly from the experimental chamber and overflowing through a sampling bottle. This source of error has been recognized of course, and in some investigations the experimental chamber has been covered in attempts to minimize stimulation of the fish. It seems very doubtful, however, whether covering a goldfish so that it cannot see the investigator is an adequate safeguard against disturbance. One advantage of using an activity detector in the continuous flow method then lies in the fact that any sudden change in activity occurring at the time of sampling can be detected, so that the reliability of the sample may be judged. Furthermore, the activity record can be used to test the effectiveness of the steps taken to avoid disturbance.

It is of course well known that fluctuations in activity during the test periods constitute a major obstacle to the correct interpretation of measurements of oxygen consumption, and numerous attempts to overcome this difficulty have been described (Ege and Krogh, 1914; Hall, 1929; Adkins, 1930; Keys, 1930; Wells, 1932, 1935; Clausen, 1933; Smith and Matthews, 1942). These measures include the use of narcotics, observing that the fish remains quiet, maintaining constant conditions of light, sampling at the same time each day, restricting the movements of the fish, and maintaining the fish in an experimental chamber until it appears to have come to rest or at any rate to have reached a steady state. Although such measures may permit the establishment of the reality of a change in oxygen consumption in connection with an experimental procedure, they do not appear to give a completely satisfactory basis for the interpretation of that change. The interpretation must be based on knowledge of the activity of the fish, inasmuch as oxygen consumption is affected by changes in the basal metabolic rate as well as by activity. The method employed must therefore be capable of supplying information on activity and oxygen consumption at the same time, so that the fraction of the respiratory exchange associated with basal metabolism may be distinguished from that due to muscular activity (Wells, 1935). None of the above methods seems to be adequate for this purpose.

Narcotics are of doubtful value in studies of this type, even for measuring basal metabolic rate alone (Adkins, 1930). Among other objections are indications that an important fraction of the metabolic functions of the fish may be suppressed to such an extent that the oxygen consumption falls below the basal level as it is generally understood (Keys and Wells, 1930). In fact, Ege and Krogh (1914) considered it necessary to use artificial respiration to insure the survival of their goldfish, the narcotic having interfered with normal respiratory movements. The other methods are open to criticism because they are based on the assumption, rather than the knowledge, that the fish is quiescent or at a constant level of activity under the conditions of the experiment. The results of the present work suggest that for the goldfish at any rate this assumption may be unwarranted. The fact that a goldfish is quiescent while it can be seen should not be taken as proof that it remains so while unobserved, and it does not seem justifiable to assume that constant environmental conditions mean constant rates of activity. So far as the goldfish is concerned, the individual variations in activity open to question the reliability of methods based on sampling at the same time each day, particularly if several fish are being compared. Confining the fish to a small respiration chamber to restrict its movements gives no assurance that it will remain quiescent or even at a constant rate of activity, and the fact that the oxygen consumption varies over a wide range in such chambers supports this objection. This method would seem to have a further disadvantage for measuring the basal metabolic rate in that a fish confined to a small tube must swim continuously, however slowly, in order to maintain its position in the current. The practice of leaving the fish in the respiratory chamber until its oxygen consumption has reached a relatively low and constant rate (Keys, 1930; Wells, 1935) is far superior to the earlier techniques, but it is limited in its application by the fact that it gives no information as to the amount of activity associated with the steady state.

The requirements of a satisfactory method appear to be met by combining an activity detector with the continuous flow system. The rate of activity can then be

measured at the same time that oxygen consumption is determined, and the results interpreted accordingly. As the activity record is continuous, periods of quiescence can be selected for measuring basal oxygen consumption, so that it is not necessary to employ special techniques designed to control activity. In this connection, however, starvation may be used as a means of prolonging the quiescent state. A further advantage of the present method lies in the fact that the fish can be maintained in good health in the experimental chamber for months, so that measurements of its respiratory metabolism need not be obscured by the excitement and other effects of handling.

SUMMARY

1. Apparatus for making continuous records of the activity of isolated and undisturbed goldfish is described, together with a method for measuring oxygen consumption and activity simultaneously.

2. The goldfish were quite variable in their patterns and rates of activity under the experimental conditions. Some fish were diurnally active and nocturnally quiescent, others followed the opposite pattern and still others were arrhythmic throughout the periods during which they were observed. Moreover, some fish showed both rhythmic and arrhythmic states of activity when studied for periods extending over several weeks or months.

3. Food, light and minor disturbances had pronounced effects on the activity of the goldfish.

4. Simultaneous measurements of oxygen consumption and activity are presented which indicate that the two are closely related above the basal level of oxygen consumption.

5. The bearing of these observations on the collection and interpretation of data on the oxygen consumption of the goldfish and on the measurement of its basal metabolic rate is discussed, and certain advantages of the method are described.

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